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13. Abstract ***(Maximum 200 Words)*** *(abstract should contain no proprietary or confidential information)*

The intracellular polyamines, spermidine, spermine, and putrescine, play an important role in the proliferation and death of normal and malignant cells. As a consequence, our work has focused on development of inhibitors of this metabolic pathway. A phase II trial of diethylnorspermine (DENSpm) for women with advanced breast cancer was completed. No major toxicity was observed but clinical activity was not sufficient to warrant further testing of DENSpm at the dose and schedule used. Analysis of DENSpm- and control-treated breast cancer tumors derived from discarded mastectomy tissue showed that DENSpm can modulate expression of the SSAT enzyme on some specimens. This could potentially serve as a biomarker of effect The efficacy of several new polyamine ananogs as treatment for established breast cancer in a nude mouse model xenograft has been demonstrated. Future studies will focus on these agents.

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INTRODUCTION

The intracellular polyamines, spermidine, spermine, and putrescine, play an important role in the proliferation and death of normal and malignant cells. As a consequence, work has focused on development of inhibitors of this metabolic pathway. The initial purpose of these ongoing studies was to evaluate the therapeutic and preventive actions of one polyamine analog, DENSpm (N^1,N^{11}-diethylnorspermine or (bis)ethylnorspermine) in breast cancer. This was to be accomplished through four related technical objectives. They included: 1) to test the therapeutic efficacy of DENSpm against human breast cancer cell lines in nude mice, 2) to use transient organ cultures of normal and malignant human breast tissues to assess effects of DENSpm on biological and pharmacological parameters relevant to antineoplastic activity, 3) to evaluate the efficacy of DENSpm in a phase II trial in women with metastatic breast cancer; and 4) to evaluate the chemopreventive activity of DENSpm in the rat mammary tumor model.

BODY

Technical Objective 1: To test therapeutic efficacy of DENSpm against estrogen receptor-positive MCF-7 and estrogen receptor-negative MDA-MB-468 human breast cancer cell lines growing in nude mice and assess tumors for possible biological or pharmacological end-points which predict subsequent tumor response.

The nude mice studies initially proposed were ultimately altered to address the in vivo testing of four novel polyamine analogs made available by SLIL Biomedical Corporation in the MDA-MB-231 human breast cancer xenograft model. The four compounds selected included SL 11093, SL 11144, SL11241, and SL11246; they were chosen after a large screen of a number of compounds by MTT assay. All four compounds were administered as a daily intraperitoneal dose for 5 days of every 21 day cycle. Of the four compounds, SL 11144 was the most effective at delaying tumor growth and was associated with the least overt toxicity.

As a result, SL11144's mechanisms of action were studied extensively in cell culture using the MCF-7, MDA-MB-231 and MDA-MB-435 cell lines. The compound was found to inhibit proliferation and induce programmed cell death as demonstrated by morphological changes and oligonucleosomal DNA fragmentation. Detailed assessment of possible cell death pathways showed that the compound activated different apoptotic pathways in the three cell lines. These results were summarized in a presentation at the 2002 American Association of Cancer Research meeting and a manuscript detailing these studies is under review in Cancer Research.

In addition, the activity of two other polyamine analogs, CPENSpm and CHENSpm, in conjunction with standard chemotherapeutic drugs was studied in cell culture systems. Antagonism, additivity, or synergy of the combinations was determined using the median effect/combination index model. The chemotherapeutic agents chosen, cis-platinum, doxorubicin, 5-fluorouracil, fluorodeoxyuridine, paclitaxel, docetaxel, 4-hydroperoxycyclophosphamide and vinorelbine, all have antitumor activity in breast cancer and represent a spectrum of mechanisms. Three treatment schedules of polyamine analogs and cytotoxics were tested in MCF-7 and MDA-MB-468 cells, demonstrating a schedule-dependence of synergistic growth inhibition. Cytotoxic agents alone for 24 hours followed by polyamine analog alone for 96 hours resulted in the most synergistic combinations and the greatest synergy. This schedule was also tested in three additional breast cancer cell lines. Two cytotoxics, vinorelbine and the fluoropyrimidines, showed the most promise

in combination with polyamine analogs. They were able to synergize with one or both polyamine analogs in most of the breast cancer cell lines. These preclinical data demonstrated a treatment schedule and combination of polyamine analogs and cytotoxics that will be important to study mechanistically and clinically for breast cancer. This work was published in Hahm et al, Clinical Cancer Research 7:391-9, 2001.

Technical Objective 2: To use transient organ culture of normal and malignant human breast tissues to assess the effects of DENSpm on biological and pharmacological parameters relevant to antineoplastic activity as identified in Technical Objective 1.

For these studies normal and malignant breast tissues obtained from discarded mastectomy specimens were incubated with or without 10 uM DENSpm for 24 hours in a transient organ culture system. Tissues were then harvested and fixed for subsequent immunohistochemical studies for SSAT. Low levels of SSAT expression were seen in some normal mammary epithelial cells adjacent to invasive cancer or in paired normal specimens. Table 1 shows that variable amounts of SSAT expression were seen after 24 hours of treatment. Presumably this would translate into differential effects of DENSpm on the cells with long-term treatment although this cannot be ascertained from this type of short-term assay. However, these data clearly demonstrated that SSAT expression in human tissues can be modulated in some cases by exposure to DENSpm; it could potentially serve as an intermediate biomarker.

Table 1: SSAT expression in primary human breast cancer explants after DENSpm.
Explants from primary human breast cancer were incubated overnight in the presence or absence of 10 μM DENSpm. Samples were fixed and paraffin-embedded sections were stained with anti-SSAT antibody as previously described. SSAT expression was quantitated on a 0-3+ scale.

Histology	No. of cases	3+	2+	1+	0
Infiltrating ductual	18	4	4	7	3
DCIS	2	1	1	0	0
Infiltrating lobular	3	0	1	2	0
Total	23	5	6	9	3

Technical Objective 3. To evaluate the efficacy of DENSpm in a phase II trial in women with metastatic breast cancer.

A total of 16 patients were enrolled on protocol J9951 (An Open-Label, Single Center, Phase 2 Study of Intravenous Diethylnorspermine DENSPM in the Treatment of Patients with Previously Treated Metastatic Breast Cancer) between April, 2000 and July, 2001. All patients had evidence of progressive metastatic breast cancer and had been treated with at least one but no more than two prior palliative chemotherapy regimens. Each cycle of therapy consisted of a 15-minute infusion of DENSpm (100 mg/m^2 daily X 5) given every 3 weeks. The primary study objective was to estimate if at least 20% of patients were progression-free after 4 months. The overall accrual goal was 34 patients (30 evaluable) with a two-stage design. The second stage of accrual was to proceed if two or more patients among the first 15 evaluable patients were

progression-free after 4 months. A total of 16 evaluable patients received 43 cycles of therapy (median = 2, range 1-6 cycles). Six of 16 patients had stable disease after 2 cycles and 1 of 16 patients had stable disease after 4 cycles. No patient had stable disease after 4 months. Thus the trial was closed to further accrual.

Therapy was extremely well tolerated. There was no significant hematological toxicity. Mild perioral numbness during the infusion was commonly seen, especially in cycle 1. Two patients required hospitalization during the second cycle of therapy for grade 3 abdominal pain that resolved with conservative management; both continued therapy with a 20% dose reduction. No neurological toxicity was observed.

In summary, DENSpm was well tolerated. No patients were free of disease progression after 4 months of treatment. Thus the study did not proceed to the second stage of accrual. However, successful completion of the first phase of this trial provides encouragement for evaluation of other polyamine analogs for treatment and prevention of breast cancer. The results of this study were presented at the 2001 San Antonio Breast Cancer Symposium.

Technical Objective 4: To evaluate the chemopreventive activity of DENSpm in the DMBA rat mammary tumor model.

Implementation of this technical objective has not yet been undertaken. Given the lack of activity of DENSpm in the clinical trial and our wish to screen other polyamine analogs, especially the SLIL compounds, for treatment efficacy, these studies were delayed until results were available from Technical Objective 1. It is anticipated that they will move forward as part of a no–cost extension of this grant if permission is granted by the DOD Breast Cancer Program.

KEY RESEARCH ACCOMPLISHMENTS

- Completion of a phase II trial to assess the efficacy of DENSpm in women with advanced breast cancer
- Demonstration that short-term exposure to DENSpm can induce expression of the SSAT protein in human breast cancer organ cultures
- Identification of novel polyamine analogs that induce programmed cell death in human breast cancer cell lines and elucidation of those death pathways
- Definition of combinations of standard cytotoxic agents and polyamine analogs that inhibit growth of human breast cancer cell lines in a synergistic fashion

REPORTABLE OUTCOMES

Publications/manuscripts submitted or published

1. Hahm HA, Dunn VR, Butash KA, Deveraux WL, Woster PM, Casero Jr RA, and Davidson NE. Combination of standard cytotoxic agents with polyamine analogues in the treatment of breast cancer cell lines. Clinical Cancer Res 7:391-99, 2001.
2. Huang Y, Yager ER, Phillips DL, Dunn VR, Hacker A, Frydman B, Valasinas AL, Reddy VK, Marton LJ, Casero RA and Davidson NE. A novel polyamine analogue inhibits growth and induces apoptosis in human breast cancer cells (submitted).

Abstracts/presentations

1. Wolff AC, Bowling MK, Declue C, Armstrong DK, Fetting JH, Casero Jr RA, and Davidson NE. A phase II study of diethylnorspermine (DENSPM) in previously treated patients with metastatic breast cancer (MBC). Breast Cancer Res Treat 69:286, 2001.
2. Huang Y, Hager ER, Phillips DL, Hacker A, Frydman B, Valasinas AL, Reddy VK, Marton LJ, Casero RA, and Davidson NE. Conformationally constrained polyamine analogues and oligoamines inhibit growth and induce apoptosis in human breast cancer cells. Proc Amer Assoc Cancer Res 43:90, 2002.

Grant applications submitted or awarded

Awarded

1. Project 3C of Breast Cancer SPORE at Johns Hopkins (NIH CA 88843) funded from Sept 30, 2000-Sept 29, 2005. Nancy E. Davidson SPORE Director and Robert Casero Jr, Project Leader for Project 3C

Under review

2. Application entitled "Polyamine analogs for chemoprevention of ER-negative breast cancer" submitted in response to NIH RFA CA-03-005 on July 25, 2002. Nancy Davidson (PI) with co-investigators Robert Casero Jr and Edward Gabrielson.
3. Application for a postdoctoral fellowship entitled "Antineoplastic efficacy of a novel polyamine analogue in human breast cancer". Submitted to the Department of Defense Breast Cancer Program on June 14, 2002. Yi Huang M.D, Ph.D. (PI).

CONCLUSIONS

A series of laboratory and clinical studies to evaluate the effects of several different polyamine analogs on growth of human breast cancer cells in nude mouse models, transient organ cultures derived from human breast cancer tissues, and women with breast cancer has been undertaken. One analog, DENSpm, was found to modulate the expression of a critical polyamine analog catabolic enzyme, SSAT, as judged by immunohistochemistry performed on short-term organ cultures derived from human breast cancer specimens. A phase II trial of DENSpm in women with advanced breast cancer showed that the drug was well tolerated but did not have sufficient clinical activity using the dose and schedule chosen to warrant further testing.

Preclinical studies have identified a new family of polyamine analogs that have excellent activity in human breast cancer cell lines in vitro and in vivo. These will be evaluated in future studies.

REFERENCES

None

APPENDICES

One reprint, one manuscript, and two abstracts.

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Publications/manuscripts submitted or published

Hahm HA, Dunn VR, Butash KA, Deveraux WL, Woster PM, Casero Jr RA, and Davidson NE. Combination of standard cytotoxic agents with polyamine analogues in the treatment of breast cancer cell lines. Clinical Cancer Res 7:391-99, 2001.

Huang Y, Yager ER, Phillips DL, Dunn VR, Hacker A, Frydman B, Valasinas AL, Reddy VK, Marton LJ, Casero RA and Davidson NE. A novel polyamine analogue inhibits growth and induces apoptosis in human breast cancer cells (submitted).

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PERSONNEL SUPPORTED

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Combination of Standard Cytotoxic Agents with Polyamine Analogues in the Treatment of Breast Cancer Cell Lines[1]

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ABSTRACT

Polyamines are essential for cell growth and differentiation. Structural polyamine analogues have been shown to have antitumor activity in experimental models including breast cancer. The ability of polyamine analogues to alter activity of cytotoxic chemotherapeutic agents in breast cancer models has not been evaluated. This study evaluates the ability of two polyamine analogues, N^1-ethyl-N^{11}-[(cyclopropyl)methyl]-4,8-diazaundecane (CPENSpm) and N^1-ethyl-N^{11}-[(cycloheptyl)methyl]4,8-diazaundecane (CHENSpm) to synergize with cytotoxics in five human breast cancer cell lines. Antagonism, additivity, or synergy of the combinations was determined using the median effect/combination index model. The chemotherapeutic agents chosen, *cis*-diaminechloroplatinum(II), doxorubicin, 5-fluorouracil, fluorodeoxyuridine, 4-hydroperoxycyclophosphamide, paclitaxel, docetaxel, and vinorelbine, all have antitumor activity in breast cancer and represent a spectrum of mechanisms. Three treatment schedules of polyamine analogue and cytotoxic were tested in MCF-7 and MDA-MB-468 lines, demonstrating a schedule-dependence of synergistic growth inhibition. Cytotoxic agent alone for 24 h followed by polyamine analogue alone for 96 h resulted in the most synergistic combinations and the greatest synergy. This schedule was then tested in three additional breast cancer lines, and several synergistic combinations were again identified. Two cytotoxics, vinorelbine and the fluoropyrimidines, showed the most promise in combination with the polyamine analogues. They were able to synergize with one or both polyamine analogues in most of the breast cancer cell lines. CPENSpm was also able to synergize with virtually all of the cytotoxics in the estrogen receptor α-positive MCF-7 and T-47D lines. These preclinical data demonstrate a treatment schedule and combinations of polyamine analogues and cytotoxics that will be important to study mechanistically and clinically for breast cancer.

INTRODUCTION

Polyamines are essential for cell growth and differentiation and the finding that polyamine levels are increased in malignant *versus* normal tissues (1–3) has implicated the polyamine metabolic pathway as a target for antineoplastic therapy (2, 4, 5). Investigators have synthesized structural analogues that can mimic the natural polyamines in their self-regulatory role, yet are unable to substitute for polyamines in terms of supporting cell growth and differentiation (5, 6). These analogues have been shown to have antitumor activity in multiple experimental model systems including breast cancer (7–14). *In vitro* growth of several breast cancer cell lines is inhibited by several spermine analogues including the *n*-alkylated, symmetrically substituted analogues, DESpm[5] (also known as BESpm) and DENSPM (also known as BENSpm), and the unsymmetrically substituted compounds, CPENSpm and CHENSpm (9, 14). In addition, several of these analogues induce PCD in breast cancer cell lines (14).

The polyamine analogue DENSPM has been evaluated in Phase I clinical trials (15, 16). Using a once-daily infusion schedule (for 5 days, repeated every 21 days), the drug was well tolerated, and gastrointestinal toxicity was the dose-limiting toxicity. There was no significant hematological toxicity, and this schedule is currently being evaluated in Phase II studies. Pharmacokinetic analysis using this dosing schedule demon-

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[5] The abbreviations used are: DESpm, N^1,N^{12}-diethylspermine (also known as BESpm, N^1,N^{12}-bis(ethyl)spermine); DENSPM, N^1-N^{11}-diethylnorspermine (also known as BENSpm, N^1,N^{11}-bis(ethyl)norspermine); CPENSpm, N^1-ethyl-N^{11}-[(cyclopropyl)methyl]-4,8-diazaundecane and CHENSpm, N^1-ethyl-N^{11}-[(cycloheptyl)methyl]4,8-diazaundecane; BESpd,N^1,N^8 bis(ethyl)spermidine, c-DDP, *cis*-diaminechloroplatinum(II); 5-FU, 5-fluorouracil; FdURd, fluorodeoxyuridine; 4HC, 4-hydroperoxycyclophosphamide; DFMO, difluoromethylornithine; MTT, 3-(4,5-dimethyl-2-yl)-2,5-diphenyl tetrazolium bromide; CI, combination index; PCD, programmed cell death; SSAT, spermidine/spermine N^1-acetyl-transferase; MGBG, methylglyoxal-bis-(guanylhydrazone).

strated patient plasma concentrations in the micromolar range that are consistent with concentrations required *in vitro* for inhibition of cell growth and induction of PCD. The antitumor activity of polyamine analogues in multiple experimental model systems, as well as the preliminary clinical data available for the analogue DENSPM, attest to the therapeutic potential of this class of agents.

Although all of the roles of polyamines in cell proliferation are not known, the capacity to interact with DNA (17) and affect DNA conformation (18) are thought to play a role in their normal cellular function. Therefore, several investigators have evaluated whether depletion of polyamine pools can modulate the activity of DNA-reactive drugs in tumor model systems. These studies have generally combined chemotherapeutic agents with compounds that deplete polyamine pools via inhibition of key biosynthetic enzymes such as ornithine decarboxylase or *S*-adenosylmethionine decarboxylase (19–36). The results of these experiments have been mixed, with some combinations demonstrating synergistic or additive activity and others demonstrating antagonism.

Only two combination studies have been done in breast cancer models. Thomas and Kiang (36) evaluated the activity of antiestrogens in combination with DFMO, an inhibitor of ornithine decarboxylase in the breast cancer cell line MCF-7 and demonstrated additive activity of these agents on cell growth inhibition. Das *et al.* (35) showed that concomitant or pre- or post-paclitaxel exposure of MCF-7 cells to DFMO resulted in antagonism of paclitaxel-induced cell growth inhibition and apoptosis.

Combined effects of cytotoxic chemotherapeutic agents and polyamine analogues in preclinical models of breast cancer have not been assessed. In this report, we evaluate the activity of the polyamine analogues, CPENSpm and CHENSpm, in combination with multiple cytotoxic chemotherapeutic agents currently in use in the treatment of breast cancer in breast cell lines, *in vitro*.

MATERIALS AND METHODS

Compounds, Cell Lines, and Culture Conditions. CPENSpm and CHENSpm were synthesized as described previously (37). For all of the experiments, a concentrated solution (10 mM in water, stored at −20°C) was diluted in medium to desired concentration. 5-FU, c-DDP, and vinorelbine were obtained from the Johns Hopkins Oncology Center pharmacy. 5-FU and c-DDP were stored at −20°C, and vinorelbine at 4°C. Paclitaxel (a gift from Bristol-Myers/Squibb) was stored at 4°C as a 10-mM solution in DMSO. Docetaxel, a gift from Rhone Poulenc/Rorer, was stored at −20°C in absolute ethanol. 4HC was a gift from Dr. O. Michael Colvin (Duke Cancer Center, Durham, NC). 4HC was stored in powder form at −20°C and dissolved in fresh cell culture medium immediately prior to its use. FdURd and doxorubicin were obtained from the Sigma Co. and stored at −20°C in water and DMSO, respectively. All of the drugs were diluted in cell culture medium to desired final concentrations except for docetaxel, which was initially diluted in water and then in cell culture medium. The acquisition and maintenance of the breast cancer cell lines, MCF-7, T-47D, MDA-MB-468, Hs 578T, and MDA-MB-231, have been previously described (38).

Growth Inhibition Assays. Cells in the exponential growth phase were plated at 1–5 × 10^4 cells/cm^2 in 24-well tissue culture plates. After attachment overnight, the medium was changed, and the cells were incubated with or without drugs for the desired exposure times. After 120 h, the cells were detached by trypsinization and counted using a Coulter counter. Percentage growth inhibition was determined by comparison of cell number per well in treated *versus* control cells.

Growth inhibition was also assessed using the MTT (Sigma Chemical Co.) dye assay (39). For the MTT assay, cells were plated in 96-well dishes and treated as above. On completion of the treatment period, the media was discarded, and 100 μl of MTT (5 mg/ml in culture medium filter sterilized) was added to each well for 4 h at 37°C. The MTT solution was then removed, and the formazan crystals were dissolved in 200 μl/well of a 1:1 (v/v) solution of DMSO:ethanol for 20 min at ambient temperature. Change in absorbance was determined at $A_{540\ nm}$. Results were compared with wells that contained culture medium but no cells, and percentage growth inhibition was calculated by comparison of the $A_{540\ nm}$ reading from treated *versus* control cells. Drug concentrations that resulted in an IC_{50} were determined from the plots of percentage growth inhibition *versus* the logarithm of the drug concentration. All of the experiments were plated in triplicate wells and were carried out at least twice. Prior to the usage of the MTT assay in experiments, the results were validated by direct comparison of results from MTT assay and conventional cell growth assays; results were consistently comparable.

Clonogenic Assay. For colony formation assay, 200 MCF-7 cells per 60-mm tissue culture dish were allowed to attach overnight. The chemotherapeutic drug of interest was then added on day 0. After 24 h, media were removed, and the cell monolayer washed with drug- and serum-free media. The cells were then exposed for the remainder of the culture period to media containing the polyamine analogue alone. After 10 days, the cell monolayer was washed once with PBS, stained with crystal violet [0.5% crystal violet in a 3:1 (v/v) mixture of water to methanol], washed with water, and allowed to dry at ambient temperature. Visible colonies were counted.

Synergy Studies. The median effect/CI Analysis (40) was used to determine antagonism, additivity, or synergy of combination exposures to both polyamine analogues and cytotoxic drugs. Cell cultures were treated with each agent individually at its IC_{50} concentration and at fixed multiples (two and three times) and fractions (0.75, 0.50, and 0.25) of the IC_{50} concentrations. The agents (polyamine analogue and drug) were also combined in these same dose-fixed ratios to determine CI. Antagonism was defined as any CI value above 1, additivity as CI = 1, and synergy as $<1 \pm$ SD. Experiments were done in triplicate, and each experiment yielded one CI value. Experiments that yielded a CI of less than 1 were repeated at least three times to allow for determination of SD for the CI values obtained. Experiments that yielded CI values of >1 were repeated once if the results were consistent, and the CI value shown is a representative value from one of these experiments. CI values are shown only for fractional growth inhibition levels of 0.50 or

greater, because dose intensity is known to be important in breast cancer treatment (41).

Treatment Schedules. Three different treatment schedules were used to mimic schedules that are potentially clinically relevant. The first treatment schedule used simultaneous exposure to both polyamine analogue and cytotoxic drug for 120 h. In the second treatment schedule, the cells were exposed to 24 h of cytotoxic drug (starting on day 0). The medium was then discarded, the cell monolayer washed once with drug-free medium, and fresh medium containing the polyamine analogue was added for the remainder of the culture period (96 h). The third treatment schedule evaluated cell exposure to polyamine analogue alone for 24 h followed by removal of the medium and addition of medium containing both polyamine analogue and drug for the remainder of the culture period (96 h). Sustained exposure to the polyamine analogue was used in all of the treatment schedules because other studies have shown that lengthy exposure is necessary for optimal polyamine analogue activity (5).

Analysis of Polyamine Content. The polyamine content of treated and untreated cells was determined by precolumn dansylation, reversed-phase, high-performance liquid chromatographic methods of Kabra *et al.* (42).

RESULTS

The estrogen receptor positive, wild-type p53 MCF-7 cells and the estrogen receptor negative, mutant p53 MDA-MB-468 cells were chosen for these studies because they are representative of hormone-dependent and -independent breast cancer cells. Six chemotherapeutic agents (c-DDP, doxorubicin, 5-FU, vinorelbine, paclitaxel, and docetaxel) were tested in combination with the two polyamine analogues (CPENSpm and CHENSpm) in both lines using the three different treatment schedules. In addition, FdURd and 4HC were tested using the treatment schedule of cytotoxic drug followed by polyamine analogue.

Effects of CPENSpm or CHENSpm and Chemotherapeutic Drugs on MCF-7 Cells. The schedule of drug exposure for 24 h followed by CPENSpm for 96 h in MCF-7 cells showed a synergistic effect on growth for all of the eight cytotoxic drugs at a fractional growth inhibition of 0.50 or greater (Table 1). The greatest degree of synergy was seen with the fluoropyrimidines and vinorelbine. In contrast, concurrent exposure to drug and CPENSpm for 120 h resulted in synergistic growth inhibition only with 5-FU and vinorelbine at fractional growth inhibition of 0.75. Similarly, the schedule of CPENSpm, followed by CPENSpm and drug, led to synergistic growth effects only with doxorubicin, c-DDP, paclitaxel, and docetaxel. The degree of synergy and the range of fractional growth inhibitions for which synergy was seen were also less with this sequence than those seen with the schedule of drug exposure followed by CPENSpm.

Because these studies were performed using the MTT assay, similar studies were performed using the colony formation assay rather than growth inhibition as an end point to validate the MTT assay (data not shown). Agreement between results of the clonogenic assay and the growth inhibition studies was seen, thereby validating the use of the MTT assay.

Table 1 Effects of CPENSpm and chemotherapeutic drugs on MCF-7 cells[a]

Drug	Fractional growth inhibition[b] 0.50	0.75	0.90
Concomitant CPENSpm + drug			
Doxorubicin	1.13 ± 0.16	1.04 ± 0.18	0.99 ± 0.27
c-DDP	1.08 ± 0.13	0.95 ± 0.18	0.88 ± 0.22
5-FU	0.69 ± 0.42	0.75 ± 0.23	1.27 ± 0.62
Vinorelbine	0.73 ± 0.37	0.67 ± 0.29	0.68 ± 0.27
Paclitaxel	1.16 ± 0.11	1.12 ± 0.14	1.12 ± 0.16
Docetaxel	0.93 ± 0.07	0.83 ± 0.25	0.80 ± 0.38
Drug then CPENSpm			
Doxorubicin	0.84 ± 0.08	0.65 ± 0.09	0.51 ± 0.08
c-DDP	0.85 ± 0.11	0.69 ± 0.13	0.63 ± 0.19
5-FU	0.58 ± 0.13	0.49 ± 0.12	0.42 ± 0.12
FdUrd	0.67 ± 0.16	0.54 ± 0.08	0.47 ± 0.11
4HC	0.93 ± 0.12	0.77 ± 0.16	0.67 ± 0.22
Vinorelbine	0.30 ± 0.29	0.32 ± 0.20	0.43 ± 0.06
Paclitaxel	0.74 ± 0.14	0.68 ± 0.11	0.67 ± 0.05
Docetaxel	0.82 ± 0.15	0.66 ± 0.19	0.54 ± 0.21
CPENSpm then CPENSpm and drug			
Doxorubicin	1.08 ± 0.27	0.88 ± 0.18	0.76 ± 0.13
c-DDP	1.17 ± 0.19	0.84 ± 0.07	0.64 ± 0.07
5-FU	0.88 ± 0.14	0.81 ± 0.19	0.85 ± 0.42
Vinorelbine	0.74 ± 0.33	0.77 ± 0.27	0.89 ± 0.37
Paclitaxel	0.99 ± 0.13	0.82 ± 0.14	0.69 ± 0.15
Docetaxel	0.69 ± 0.29	0.58 ± 0.20	0.50 ± 0.15

[a] Results are shown for combination studies using the polyamine analog, CPENSpm, and drug at three different treatment schedules.

[b] CI values for fractional growth inhibitions of 0.50, 0.75, and 0.90 in the MCF-7 cell line. Antagonism CI > 1.00; additivity CI = 1.00; synergy CI < 1.00 ± SD.

Identical studies were undertaken using CHENSpm and cytotoxics in MCF-7 cells (Table 2). Evidence for synergistic interaction was seen only with the sequence of cytotoxic agent followed by CHENSpm. Unlike CPENSpm, synergy was seen with fewer drugs including c-DDP and paclitaxel at a fractional growth inhibition of 0.90, 5-FU and FdURd at a fractional growth inhibition of ≥0.75, and vinorelbine at a fractional growth inhibition of ≥0.50. No synergistic combinations were seen with the treatment schedule of concurrent treatment or CHENSpm followed by the combination of cytotoxic and CHENSpm.

Endogenous Polyamine Levels and Analogue Levels in Combination Studies in the MCF-7 Cells. A key question is whether the observed growth inhibitory effects of combinations of polyamine analogues and cytotoxics simply reflects effects on intracellular polyamines or on polyamine analogue levels. Therefore, the effect of cytotoxic drugs for 24 h followed by analogue alone for the remainder of the culture period on polyamine levels and analogue levels was assessed. Cells were harvested on day 5 for measurement of polyamines and analogues. In all of the experiments, CHENSpm alone did not substantially perturb endogenous polyamine pools, whereas CPENSpm treatment resulted in depletion of spermidine and spermine. Studies using paclitaxel, docetaxel, and 4HC in com-

Table 2 Effects of CHENSpm and chemotherapeutic drugs on MCF-7 cells[a]

Drug	Fractional growth inhibition[b] 0.50	0.75	0.90
Concomitant CHENSpm + drug			
Doxorubicin	1.18 ± 0.15	1.08 ± 0.22	1.05 ± 0.25
c-DDP	1.37 ± 0.61	1.14 ± 0.56	1.10 ± 0.44
5-FU	1.28 ± 0.13	1.11 ± 0.02	1.00 ± 0.08
Vinorelbine	1.11 ± 0.22	1.02 ± 0.14	0.96 ± 0.11
Paclitaxel	1.22 ± 0.09	1.27 ± 0.06	1.34 ± 0.02
Docetaxel	1.19 ± 0.14	1.21 ± 0.10	1.27 ± 0.04
Drug then CHENSpm			
Doxorubicin	1.17	1.28	1.43
c-DDP	1.33 ± 0.38	1.38 ± 0.36	0.87 ± 0.11
5-FU	1.06 ± 0.06	0.87 ± 0.03	0.73 ± 0.02
FdUrd	0.92 ± 0.27	0.55 ± 0.13	0.45 ± 0.06
4HC	1.11 ± 0.18	0.91 ± 0.18	0.78 ± 0.22
Vinorelbine	0.77 ± 0.15	0.64 ± 0.10	0.54 ± 0.07
Paclitaxel	1.15 ± 0.20	0.95 ± 0.15	0.81 ± 0.15
Docetaxel	1.19	1.31	1.44
CHENSpm then CHENSpm and drug			
Doxorubicin	1.36 ± 0.12	1.22 ± 0.06	1.22 ± 0.06
c-DDP	1.30	1.13	1.06
5-FU	1.44	1.19	1.04
Vinorelbine	1.43 ± 0.08	1.29 ± 0.05	1.20 ± 0.12
Paclitaxel	1.39 ± 0.11	1.36 ± 0.11	1.36 ± 0.18
Docetaxel	1.54	1.55	1.64

[a] Results are shown for combination studies using the polyamine analogue CHENSpm and drug with the MCF-7 cell line at three different treatment schedules.

[b] CI values for fractional growth inhibitions of 0.50, 0.75, and 0.90 in the MCF-7 cell line. Antagonism CI > 1.00; additivity CI = 1.00; synergy CI < 1.00 ± SD.

bination with CHENSpm or CPENSpm showed that these drugs had no effect on polyamine levels alone or in combination with either analogue (data not shown). Table 3 shows the results of similar studies with the fluoropyrimidines, doxorubicin, c-DDP, and vinorelbine. None of these drugs altered polyamine levels when used alone. In contrast, 5-FU or FdURd in combination with CHENSpm or CPENSpm resulted in elevations in both analogue levels compared with treatment with CHENSpm or CPENSpm alone. CHENSpm levels were increased 3- to 6-fold, but this increase was not associated with substantial changes in the polyamine pools. CPENSpm levels increased only 1.1- to 3.5-fold, but these changes were consistently associated with further reduction in all of the three polyamine levels compared with analogue alone. For the combination studies with doxorubicin, c-DDP, and vinorelbine, there was no consistent change in intracellular analogue levels or polyamine levels for the combination of CHENSpm plus cytotoxic when compared with changes seen with CHENSpm or drug alone. But, like fluoropyrimidines, these agents also demonstrated further depletion of polyamine pools in association with a 1.2- to 2.0-fold increase in CPENSpm intracellular concentrations when combined with CPENSpm. It should be noted that the increase in CPENSpm accumulation is offset by an almost exact reduction in charge-complement of the natural polyamines as observed previously by Bergeron *et al.* (43).

Effects of CPENSpm or CHENSpm and Chemotherapeutic Drugs on MDA-MB-468 Cells. Similar studies were carried out using the estrogen receptor-negative MDA-MB-468 cell line. No evidence for synergy was seen for any treatment schedule or with any chemotherapeutic drug in combination with CPENSpm in this cell line (Table 4). Studies with CHENSpm demonstrated synergy only with treatment with either fluoropyrimidine for 24 h followed by CHENSpm for 96 h at a fractional growth inhibition of ≥0.75 for 5-FU and ≥0.90 for FdURd (Table 5).

Combination Studies Using Other Breast Cancer Cell Lines. Combination studies using the treatment schedule of cytotoxic drug for 24 h followed by CPENSpm for 96 h led to the greatest number of synergistic combinations in both MCF-7 and MDA-MB-468 cells. Therefore, this treatment strategy was evaluated in three additional breast cancer cell lines, the estrogen receptor-negative MDA-MB-231 and Hs 578T cells and the estrogen receptor-positive T-47D cells as shown in Table 6. Synergistic growth inhibition was seen with all of the drugs except doxorubicin in T-47D cells and with all of the drugs except for doxorubicin and c-DDP in Hs 578T cells. In contrast, only one combination demonstrated synergy in the MDA-MB-231 cells, CPENSpm followed by vinorelbine.

Finally, because only the two fluoropyrimidines demonstrated synergy when given before CHENSpm in MCF-7 and MDA-MB-468 cells, the sequence of 5-FU or FdURd for 24 h followed by CHENSpm for 96 h was evaluated in MDA-MB-231, Hs 578T, and T-47D cells. Synergy between 5-FU and CHENSpm was seen in all of the three lines, whereas FdURd and CHENSpm interacted in a synergistic fashion only in MDA-MB-231 and Hs 578T cells (Table 7).

DISCUSSION

Polyamine analogues have been shown to have antitumor activity as single agents in multiple experimental model systems (7–14). Their ability to modulate response to chemotherapeutic agents is worthy of study. This study addressed the activity of two polyamine analogues, CPENSpm and CHENSpm, in combination with multiple chemotherapeutic agents in breast cancer cell lines. The chemotherapeutic agents used were selected because they: (*a*) have antitumor activity in breast cancer; (*b*) are currently in use in the treatment of breast cancer; and (*c*) represent a broad spectrum of mechanisms of action. They include alkylating agents (4HC), topoisomerase II inhibitors (doxorubicin), antimetabolites (5-FU and FdURd), antimitotic agents (vinorelbine, paclitaxel, and docetaxel), and the DNA-reactive agent, c-DDP, which causes both intra- and interstrand DNA adducts.

Synergistic combinations were identified using one or both of the polyamine analogues in all of the cell lines evaluated. There was a schedule dependence for synergy, with the sequence of cytotoxic drug exposure for 24 h followed by polyamine analogue for 96 h resulting in the greatest number of synergistic combinations as well as the greatest magnitude of synergy for the MCF-7 and MDA-MB-468 cell lines. It is unclear why this schedule is superior even when using diverse

Table 3 Polyamine and polyamine analogue levels[a] on day 5 in the MCF-7 cell line

Cells were treated with drug alone on day 0 for 24 hours; then the medium was removed and the cell monolayer washed with drug- and serum-free medium. Then medium with or without analog was added for the remainder of the culture period (120 h).

Treatment groups	Putrescine nmol/mg protein	Spermidine	Spermine	CHENSpm	CPENSpm
Control	2.75	26.18	17.05	0	0
5-FU	2.21	29.15	24.96	0	0
CHENSpm	1.96	28.57	23.82	5.66	0
5-FU then CHENSpm	1.41	16.70	21.66	21.21	0
CPENSpm	11.96	18.45	8.50	0	23.95
5-FU then CPENSpm	0	2.99	8.29	0	35.05
Control	2.19	22.49	15.64	0	0
FdUrd	1.94	16.29	17.87	0	0
CHENSpm	2.09	20.96	19.08	6.55	0
FdUrd then CHENSpm	2.43	11.43	18.82	21.28	0
CPENSpm	9.22	13.32	7.48	0	39.41
FdUrd then CPENSpm	0	1.59	4.29	0	46.10
Control	2.90	25.53	15.23	0	0
Doxorubicin	2.96	21.62	11.54	0	0
CHENSpm	2.79	26.31	14.81	1.52	0
Doxorubicin then CHENSpm	3.07	20.05	11.43	1.15	0
CPENSpm	8.11	12.31	5.64	0	19.54
Doxorubicin then CPENSpm	2.24	3.86	1.83	0	25.58
Control	2.75	26.18	17.05	0	0
c-DDP	0	20.35	22.40	0	0
CHENSpm	1.96	28.57	23.82	5.66	0
c-DDP then CHENSpm	1.19	19.60	19.92	7.38	0
CPENSpm	11.96	18.45	8.50	0	23.92
c-DDP then CPENSpm	1.31	4.62	4.85	0	32.43
Control	6.46	28.36	15.16	0	0
Vinorelbine	4.55	20.34	15.12	0	0
CHENSpm	3.17	28.86	22.05	3.51	0
Vinorelbine then CHENSpm	4.77	15.59	17.80	6.09	0
CPENSpm	12.61	15.90	7.45	0	17.87
Vinorelbine then CPENSpm	3.69	1.68	5.17	0	26.00

[a] Polyamine and analog levels are the mean of duplicate cultures from one representative experiment. All of the experiments were done at least twice and gave similar results.

chemotherapeutic agents in combination with either polyamine analogue in the multiple breast cancer cell lines. Most of the previous combination studies with DFMO and/or MGBG and chemotherapeutic agents have focused on treatment with the enzyme inhibitor initially to perturb polyamine pools before drug therapy based on the hypothesis that resultant changes in DNA conformation may allow for greater drug access. Despite its biological rationale, this schedule gave inconsistent results with some studies demonstrating synergism (21, 25, 33) for some DNA-directed agents, whereas others demonstrated antagonism (23, 25, 31, 34, 35).

Only two studies published to date have evaluated the activity of combination studies with polyamine analogues and chemotherapeutic agents in *in vitro* tumor model systems. One study (24) combined the spermidine analogue BESpd with 4′-(9-acridinylamino)methanesulfon-M anisidide (m-AMSA) in a human lung cancer cell line to evaluate the induction of topoisomerase II-dependent drug-induced cleavable DNA complexes. Unfortunately, it did not address cell growth inhibition or colony-forming ability with combination therapy *versus* drug alone. Marverti *et al.* (44) studied the effect of the spermine analogue, BESpm, and c-DDP on the growth of c-DDP-sensitive and -resistant ovarian carcinoma cells. In the c-DDP-sensitive cell line, concomitant exposure to c-DDP and BESpm demonstrated synergy, whereas the c-DDP-resistant cell line was found to be cross-resistant to BESpm. However, when the colony-forming ability was evaluated after concurrent treatment with both of the agents, there was a synergistic interaction as determined by median effect/CI analysis. Of note, c-DDP in our breast cancer models demonstrated synergy in combination with CPENSpm and CHENSpm in the MCF-7 cell line, and CPENSpm in the T-47D cell line.

CPENSpm and CHENSpm are both spermine analogues with antitumor activity and the ability to induce PCD in multiple experimental model systems, yet they apparently have different mechanisms of action. CPENSpm has been shown to superinduce the catabolic enzyme SSAT in a number of model systems (7, 10, 45, 46). This superinduction is associated with production of hydrogen peroxide, and increased oxidative stress is believed to be an important mediator in the induction of PCD by this agent in select tumor types (47). CHENSpm, however, does not superinduce this enzyme in any model system studied; yet it has significant antitumor activity and also induces PCD. In a human lung cancer model, this agent leads to a G_2-M cell cycle arrest (47) and alters tubulin polymerization (48). These agents

Table 4 Effects of CPENSpm and chemotherapeutic drugs on MDA-MB-468 cells[a]

Drug	Fractional growth inhibition[b] 0.50	0.75	0.90
Concomitant CPENSpm + drug			
Doxorubicin	1.28 ± 0.14	1.45 ± 0.19	1.73 ± 0.28
c-DDP	0.96 ± 0.46	1.33 ± 0.60	1.75 ± 1.28
5-FU	1.32 ± 0.44	1.81 ± 0.60	2.54 ± 0.97
Vinorelbine	1.15 ± 0.02	1.10 ± 0.09	1.13 ± 0.16
Paclitaxel	1.29 ± 0.22	1.37 ± 0.25	1.55 ± 0.33
Docetaxel	1.17	1.39	1.72
Drug then CPENSpm			
Doxorubicin	1.33	1.27	1.25
c-DDP	1.22	1.21	1.22
5-FU	0.98	1.19	1.45
4HC	0.90 ± 0.16	1.00 ± 0.32	1.15 ± 0.54
Vinorelbine	0.93 ± 0.23	0.83 ± 0.18	0.78 ± 0.25
Paclitaxel	0.86 ± 0.32	0.97 ± 0.07	1.36 ± 0.48
Docetaxel	1.18	1.14	1.14
CPENSpm then CPENSpm and drug			
Doxorubicin	1.06	1.43	1.95
c-DDP	1.35 ± 0.14	1.82 ± 0.10	2.56 ± 0.30
5-FU	1.53	1.64	1.79
Vinorelbine	0.98 ± 0.15	1.05 ± 0.10	1.20 ± 0.10
Paclitaxel	1.17 ± 0.28	1.52 ± 0.45	2.71 ± 2.15
Docetaxel	1.17 ± 0.26	1.38 ± 0.31	2.17 ± 1.24

[a] Results are shown for combination studies using the polyamine analog CPENSpm and drug with the MDA-MB-468 cell line at three different treatment schedules.

[b] CI values for fractional growth inhibitions of 0.50, 0.75, and 0.90 in the MDA-MB-468 cell line. Antagonism CI > 1.00; additivity CI = 1.00; synergy CI < 1.00 ± SD.

Table 5 Effects of CHENSpm and chemotherapeutic drugs on MDA-MB-468 cells[a]

Drug	Fractional growth inhibition[b] 0.50	0.75	0.90
Concomitant CHENSpm + drug			
Doxorubicin	1.41	1.45	1.51
c-DDP	1.01	1.17	1.36
5-FU	1.40 ± 0.33	1.28 ± 0.39	1.21 ± 0.44
Vinorelbine	0.97 ± 0.32	0.97 ± 0.21	0.99 ± 0.09
Paclitaxel	1.41 ± 0.14	1.35 ± 0.13	1.30 ± 0.11
Docetaxel	1.62	1.50	1.39
Drug then CHENSpm			
Doxorubicin	1.50	1.33	1.18
c-DDP	1.13	1.11	1.15
5-FU	1.12 ± 0.19	0.92 ± 0.01	0.78 ± 0.12
FdURd	0.94 ± 0.37	0.85 ± 0.25	0.85 ± 0.13
4HC	1.11 ± 0.04	0.97 ± 0.04	0.94 ± 0.12
Vinorelbine	1.16 ± 0.07	1.03 ± 0.08	0.94 ± 0.12
Paclitaxel	2.13	1.87	1.64
Docetaxel	1.14	1.20	1.26
CHENSpm then CHENSpm and drug			
Doxorubicin	1.12 ± 0.19	1.05 ± 0.13	1.03 ± 0.11
c-DDP	1.36	1.30	1.24
5-FU	1.32	1.17	1.18
Vinorelbine	1.14 ± 0.08	1.06 ± 0.10	0.99 ± 0.12
Paclitaxel	1.30	1.28	1.26
Docetaxel	0.99 ± 0.23	0.98 ± 0.13	0.98 ± 0.06

[a] Results are shown for combination studies using the polyamine analogue CHENSpm and drug with the MDA-MB-468 cell line at three different treatment schedules.

[b] CI values for fractional growth inhibitions of 0.50, 0.75, and 0.90 in the MDA-MB-468 cell line. Antagonism CI > 1.00; additivity CI = 1.00; synergy CI < 1.00 ± SD.

demonstrated different spectrums of activities in combination with chemotherapeutic drugs in the breast cancer models.

In the breast cancer cell lines studied, two classes of agents, the fluoropyrimidines and vinorelbine demonstrated the most activity in combination with CPENSpm or CHENSpm. 5-FU demonstrated synergy in combination with CPENSpm in three of the five breast cancer cell lines evaluated when drug treatment preceded polyamine analogue exposure. 5-FU and/or FdURd demonstrated synergy in combination with CHENSpm in all of the five cell lines. This class of drugs has not been previously evaluated in combination studies with polyamine analogues, although there have been several studies in epithelial tumor model systems using DFMO in combination with 5-FU with variable results (19, 32, 33).

In contrast, our results in the breast cancer cell lines with two different spermine analogues demonstrate synergistic combinations with one or both analogues in several of the breast cancer cell lines evaluated. These findings warrant further evaluation in *in vivo* models of breast cancer and examination of the possible mechanisms responsible for synergy. 5-FU is an antimetabolite with several mechanisms of action, including inhibition of thymidylate synthase and incorporation into DNA and/or RNA (49). Evaluation of cell cycle modulation, total intracellular 5-FU content, specific incorporation into DNA and RNA, and thymidylate synthase activity in the presence of the polyamine analogue are currently being explored. Because different schedules of treatment or the same schedule in a different cell line can result in different responses (additivity, antagonism, or synergy), evaluation of these changes using different treatment schedules and breast cancer cell lines with variable responses should help identify mechanisms that play a role in a synergistic response.

One of the possible mechanisms, the effect of drug on polyamine analogue cellular accumulation and polyamine pool depletion was extensively evaluated in the MCF-7 cell line. This cell line was chosen because it demonstrated synergy with all of the cytotoxic drugs evaluated in combination with CPENSpm as well as with several drugs in combination with CHENSpm using the treatment schedule of drug followed by analogue. Five of eight drugs tested led to increased CPENSpm levels in the cells treated with the combination compared with cells treated with analogue alone, and this increase was associated with further depletion of the polyamine levels. This change may well play an important role in the synergistic response seen with these drugs in combination. Whether the increased level of CPENSpm is attributable to decreased efflux or to alterations in metabolism will need to be evaluated. In addition, it will be interesting to see whether this phenomenon occurs as well in *in vivo* model

Table 6 Effects of CPENSpm and chemotherapeutic drugs on MDA-MB-231, Hs-578T, and T-47D cells

Cells were treated with drug alone on day 0 for 24 h, then the media was removed and the cell monolayer washed with drug- and serum-free medium. Then medium with CPENSpm was added for the remainder of the culture period (120 h).

Drug	Fractional growth inhibition[b] 0.50	0.75	0.90
MDA-MB-231 cells			
Doxorubicin	1.21	1.16	1.25
c-DDP	1.07	1.41	1.86
5-FU	1.04 ± 0.62	0.91 ± 0.30	1.11 ± 0.78
FdURd	1.05 ± 0.32	1.11 ± 0.27	1.58 ± 0.51
4HC	1.20 ± 0.31	1.37 ± 0.36	0.58 ± 0.44
Vinorelbine	1.09 ± 0.06	0.89 ± 0.07	0.76 ± 0.10
Paclitaxel	1.16 ± 0.09	1.08 ± 0.07	1.04 ± 0.16
Docetaxel	0.92 ± 0.22	0.86 ± 0.25	0.85 ± 0.31
Hs 578T cells			
Doxorubicin	1.06	1.15	1.38
c-DDP	1.17	1.24	1.31
5-FU	0.82 ± 0.09	0.69 ± 0.05	0.59 ± 0.04
FdURd	0.80 ± 0.29	0.54 ± 0.15	0.45 ± 0.07
4HC	1.01 ± 0.09	0.76 ± 0.16	0.64 ± 0.16
Vinorelbine	1.18 ± 0.27	0.73 ± 0.16	0.48 ± 0.06
Paclitaxel	0.95 ± 0.19	0.76 ± 0.21	0.66 ± 0.25
Docetaxel	1.15 ± 0.07	0.98 ± 0.05	0.85 ± 0.09
T-47D cells			
Doxorubicin	1.04 ± 0.11	0.83 ± 0.28	0.84 ± 0.45
c-DDP	0.95 ± 0.13	0.75 ± 0.07	0.67 ± 0.13
5-FU	0.71 ± 0.01	0.67 ± 0.09	0.69 ± 0.20
FdURd	0.84 ± 0.06	0.79 ± 0.08	0.80 ± 0.16
4HC	1.01 ± 0.13	0.73 ± 0.02	0.58 ± 0.05
Vinorelbine	1.02 ± 0.19	0.69 ± 0.13	0.55 ± 0.20
Paclitaxel	0.89 ± 0.05	0.75 ± 0.07	0.71 ± 0.16
Docetaxel	0.91 ± 0.24	0.79 ± 0.18	0.80 ± 0.17

[a] CI values for fractional growth inhibitions of 0.50, 0.75, and 0.90 in the MDA-MB-231, Hs 578T, and T-47D cell lines. Antagonism CI > 1.00; additivity CI = 1.00; synergy CI < 1.00 ± SD.

Table 7 Effects of CHENSpm and 5-FU or FdURd on MDA-MB-231, Hs 578T, and T-47D cells[a]

Cells were treated with 5-FU or FdURd alone on day 0 for 24 h, then the medium was removed and the cell monolayer washed with drug- and serum-free medium. Then medium with CHENSpm was added for the remainder of the culture period (120 h).

Drug	Fractional growth inhibition[b] 0.50	0.75	0.90
MDA-MB-231 cells			
5-FU	0.84	0.74	0.73
FdURd	0.56 ± 0.06	0.54 ± 0.21	0.67 ± 0.36
Hs 578T cells			
5-FU	0.96 ± 0.06	0.88 ± 0.05	0.81 ± 0.07
FdURd	0.92 ± 0.11	0.75 ± 0.09	0.75 ± 0.08
T-47D cells			
5-FU	0.88 ± 0.04	0.70 ± 0.05	0.63 ± 0.02
FdURd	1.04 ± 0.06	1.02 ± 0.12	1.20 ± 0.27

[a] CI values for fractional growth inhibitions of 0.50, 0.75, and 0.90 in the MDA-MB-231, Hs 578T, and T-47D cell lines. Antagonism CI > 1.00; additivity CI = 1.00; synergy CI < 1.00 ± SD.

systems as well, and how these changes might correlate with effects on tumor growth.

Similar studies of intracellular polyamines and CHENSpm levels in MCF-7 cells showed that substantial increases in CHENSpm levels occurred only in combination with the fluoropyrimidines. What role this may play in the synergy that is seen with those agents and CHENSpm remains to be defined. Synergistic responses to drug combinations may be attributable to more than one mechanism. Thus, the finding of changes in polyamine analogue levels in combination studies will need to be evaluated in several other breast cancer cell lines to see whether this relationship between synergy and increased intracellular analogue levels holds true. Not all of the synergistic drug combinations in the MCF-7 cell line were associated with changes in polyamine pools or analogue levels, which suggests that alternate mechanisms are involved in these synergistic responses with certain chemotherapeutic agents.

The combination of vinorelbine, an antimitotic agent that binds tubulin resulting in microtubule depolymerization (50), and CPENSpm demonstrates synergy in all of the breast cancer cell lines except MDA-MB-468. But when used with CHENSpm in the MCF-7 and MDA-MB-468 cell lines, vinorelbine was antagonistic in the MDA-MB-468 cell line and synergistic in the MCF-7 cell line. Polyamines may play a role in the natural dynamics of microtubules (51, 52), and some polyamine analogues have been shown to induce a G_2-M cell cycle arrest and alter tubulin dynamics (47, 48). The positive interaction between polyamine analogues and vinorelbine may be mediated by further modulation of this pathway. Of note, the taxanes, paclitaxel and docetaxel, which are known to stabilize tubulin polymerization, have been shown to synergize with the polyamine analogues as well, albeit under more limited experimental conditions.

In addition, it is noteworthy that the combination of CPENSpm with virtually all of the cytotoxics had synergistic effects in the MCF-7 and T-47D cells. Because these drugs represent a spectrum of different mechanisms of action, their extensive ability to synergize with CPENSpm is intriguing. This result implies that the analogue may be modifying a common pathway by which all of the these drugs work to produce an antitumor response. It is known that most chemotherapeutic agents induce PCD (53, 54). Polyamine analogues are also known to induce PCD in multiple tumor types including breast cancer (13, 14, 55, 56). Studies using the CPENSpm analogue in the H157 human lung cancer cell line have shown that production of oxidative stress via hydrogen peroxide production in the cell because of SSAT induction by this analogue is a component of cell death (47). Whether this pathway is involved in the synergistic response seen with combination therapy in these cell lines remains to be evaluated.

Finally, both the MCF-7 and T-47D cell lines are estrogen receptor positive and dependent on estradiol for cell growth. It will be important to determine whether other estrogen receptor

positive breast cancer cell lines also demonstrate synergy with CPENSpm and a broad spectrum of cancer chemotherapeutic agents. If this is the case, the role of estrogen receptor-dependent proliferation pathways in breast cancer cells and their association with the activity of polyamine analogues may be important to investigate.

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A Novel Polyamine Analogue Inhibits Growth and Induces Apoptosis in Human Breast Cancer Cells[1]

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Abbreviations: PCD, programmed cell death; ODC, ornithine decarboxylase; Put, putrescine; Spd, spermidine; Spm, spermine; SSAT, spermidine/spermine N^1-acetyltransferase; PAO, polyamine oxidase; MTT, 3-(4,5, -dimethyl-2-yl)-2,5,-diphenyl tetrazolium; DMSO, Dimethyl Sulfoxide; PBS, phosphate-buffered saline; IC_{50}, concentration required to inhibit cell growth by 50 %; PARP, poly ADP ribose polymerase; PAGE, polyacrylamide gel electrophoresis; DFMO, α-difluoromethylornithine.

ABSTRACT

Polyamine analogues have demonstrated considerable activity against many important solid tumor models including breast cancer. However, the precise mechanisms of anti-tumor activities of polyamine analogues are not entirely understood. The cytotoxicity of a newly developed polyamine analogue compound, SL11144, against human breast cancer was assessed. Treatment of human breast cancer cell lines in culture with SL11144 decreased cell proliferation and induced programmed cell death in a time and dose dependent manner. SL11144 also profoundly inhibited the growth of MDA-MB-231 xenografts in host nude mice without overt toxic effects. Treatment of MDA-MB-435 cells with SL11144 led to the release of cytochrome c from mitochondria into cytosol, activation of caspase-3, and PARP cleavage. SL11144 decreased Bcl-2 and increased Bax protein levels in MDA-MB-231 cells. Furthermore, AP-1 transcriptional factor family member c-Jun was up-regulated by SL11144 in MDA-MB-435 and MDA-MB-231 cells, but not in MCF7 cells. In addition, significant inhibition of ornithine decarboxylase activity and decrease in polyamine pools were demonstrated. These results suggest that the novel polyamine analogue, SL11144, shows effective antineoplastic action against human breast cancer cells *in vitro* and *in vivo*, and multiple apoptotic mechanisms are associated with its cytotoxic effect in specific human breast cancer cell lines.

INTRODUCTION

The natural polyamines (putrescine, spermidine and spermine) have been shown to be essential for cell growth. The critical role of polyamines in regulation of cell growth has led to the development of a number of inhibitors of key enzymes in the polyamine biosynthetic pathway as a therapeutic strategy (1-3). It has also been demonstrated that synthetic polyamine analogues can down-regulate polyamine biosynthesis by feedback mechanisms, but are unable to act as substitutes for natural polyamines to promote cell growth. This approach has become an important means for the study of the physiological roles of natural polyamines and a potent application for creation of new anti-neoplastic agents (4-6). Indeed several synthetic polyamine analogues have been reported to inhibit cell proliferation and induce PCD in a variety of tumor cell lines (7-11).

Apoptotic cell death is characterized by chromatin condensation, cytoplasmic blebbing, and internucleosomal DNA fragmentation, and occurs in a variety of cellular systems in response to many different stimuli (12). We have previously demonstrated that some polyamine analogues can induce programmed cell death in hormone-responsive or -unresponsive human breast cancer cells (9). However the specific programmed cell death pathways and mechanisms have not been delineated. Polyamine metabolism is highly regulated by enzymes like ODC, SSAT and PAO (13). Cell type-specific superinduction of SSAT and the subsequent depletion of natural polyamine pools have been reported in polyamine analogue-induced growth inhibition and apoptosis in some tumor cell lines (9, 14). However, in other cell lines, polyamine analogues that do not highly induce SSAT can still inhibit tumor cell growth and produce apoptosis (15, 16). These divergent results suggest

that polyamine analogue-induced cell death may result from several agent-dependent mechanisms.

SL11144 is a leading agent of a new generation of polyamine analogue designated as oligoamines, which has shown significant activity against proliferating cells (17). In this study, we have evaluated the antineoplastic efficacy of SL11144 in human breast cancer cells *in vitro* and *in vivo*. The data presented in this study suggest that SL11144 significantly inhibits growth and induces programmed cell death in human breast cancer cells.

MATERIALS AND METHODS

Compound, cell lines, and culture condition. The polyamine analogue SL11144 (Fig. 1) was provided by SLIL Biomedical Corp. (Madison, WI). A concentrated stock solution (10 mM in ddH_2O) was diluted with medium to the desired concentrations for specific experiments. Human breast cancer MDA-MB-231 and MCF7 cells were maintained in DMEM medium supplemented with 5% fetal bovine serum, 2 mM glutamine and 100 units/ml penicillin/streptomycin. MDA-MB-435 cells were maintained in IMEM medium supplemented with 5% fetal bovine serum, 2 mM glutamine and 100 units/ml penicillin/streptomycin. Cells were incubated at 37°C in a 5% CO_2 atmosphere.

MTT survival assays. MTT assays were performed using a method described previously (18). Briefly, 2,000-5,000 cells were plated in 96-well dishes and treated with the various concentrations of SL11144 for different lengths of time. At the end of each time point, 100 µl of a 1 mg/ml MTT solution (Sigma Chemical CO.), diluted in serum free culture media, was added to each well. The plates were incubated at 37°C in 5% CO_2 atmosphere for 4 h, allowing viable cells to reduce the yellow tetrazolium salt into dark blue formazan crystals. At the end of the 4-h incubation, the MTT solution was removed and 200 µl of 1:1(v/v) solution of DMSO: ethanol was added to each well to dissolve the formazan crystals. The absorbance in individual wells was determined at $A_{540\,nm}$. All of the experiments were plated in quadruplicate and the results of assays were presented as means ± S.D.

Analysis of intracellular polyamine pools, SSAT activity, and ODC activity. The intracellular polyamine content of treated and untreated cells was determined by precolumn

dansylation and reversed phase high-performance liquid chromatography (19). SSAT and ODC activities were measured using cellular extracts as described previously (20, 21). Protein concentrations were determined according to the method of Bradford (22).

Hoechst staining of nuclear chromatin. SL11144-treated cells were fixed with 4% formaldehyde in phosphate-buffered saline (PBS) at 37°C for 10 min and permeabilized with a 19:1 mixture of ethanol/acetic acid at –20°C for 15 min. Fixed cells were stained with 1 μg/ml Hoechst 33258 (Sigma Chemical CO., St. Louis, MO) in PBS at room temperature for 20 min. Hoechst staining of the cells was analyzed by fluorescence microscopy.

Determination of internucleosomal DNA cleavage. After tumor cells were treated with increasing concentrations of SL11144 for increasing times, cells were harvested, counted, and washed with phosphate-buffered saline (PBS) at 4°C. Cells were then suspended in lysis buffer (5 mM Tris-HCL, 20 mM EDTA and 0.5% Triton X-100) and incubated for 20 min on ice. After incubation samples were centrifuged at 14,000 x g for 20 min, the supernatant was transferred to a reaction tube followed by phenol/chloroform/isoamyl (25:24:1) extraction. Two volumes of 100% EtOH was added to supernatant followed by 5 min centrifugation at 14,000 x g. The pellet was resuspended in 0.1x SSC buffer and incubated with RNase for at least 30 min at 37°C. Then 50 μl 5M NaCl was added followed by phenol/chloroform/isoamyl (25:24:1) extraction. After EtOH precipitation and centrifugation, the pellet was washed with 70% EtOH and dried. DNA samples were analyzed by electrophoresis in a 1.2% agarose slab gel containing 0.2 μg/ml ethidium bromide, and

visualized under UV illumination. This method isolates only DNA ladder fragments without genomic DNA.

Animal Studies. Four to six week old female BALB c nu/nu athymic nude mice (Harlan Bioproducts for Science Inc., Indianapolis, IN) weighing between 25 and 30 g were used for the MDA-MB-231 tumor cells xenografts. Tumor fragments were implanted into the flanks of mice and allowed to grow to a volume of 0.1-0.2 cm^3. Animals were then randomly assigned to receive vehicle control or SL11144 (10 mg/kg/mouse/day) via intraperitoneal injections for 5 consecutive days in each 3-week cycle for a total of 3 cycles. Tumor volumes were regularly assessed twice weekly by measuring $\pi/6$ x Length (cm) x Width (cm) x Width (cm) using a caliper. Mice were weighed twice weekly. All animal studies were conducted in accordance with the guidelines of the National Institutes of Health Guide for the Care and Use of Animals and the protocol was approved by the Johns Hopkins Medical Institution Animal Care and Use Committee.

Nuclear and Cytoplasmic Extraction. The extractions of nuclear and cytoplasmic protein were performed using the NE-PER™ Nuclear and Cytoplasmic Extraction Kit (Pierce, Rockford, IL). MDA-MB-435 cells treated with 10 μM SL11144 for different times were harvested by trypsinization and washed with phosphate-buffered saline (PBS). 200 μl of ice-cold Cytoplasmic Extraction Reagent I (CER I) with protease inhibitors (0.5 mg/ml Benzamidine, 2 μg/ml Aprotinin, 2 μg/ml Leupeptin, 0.2 M PMSF) was added to the cell pellets. After 10 min incubation on ice, 11 μl of ice-cold Cytoplasmic Extraction Reagent II (CER II) without protease inhibitors was added followed by 5 min centrifugation at 14,000 x

g. The supernatant containing the cytoplasmic extract was retained and the insoluble pellet was resuspended in 100 µl Nuclear Extraction Reagent (NER) and incubated on ice for 40 min. After 10 min centrifugation, the supernatant which contained the nuclear extract was saved. Both cytoplasmic and nuclear extracts were analyzed by Western blot using anti-c-Jun and anti-c-Fos antibodies as described below.

Detection of cytochrome c release. To avoid artifacts due to mechanical breakage of the outer mitochondrial membrane, selective plasma membrane permeabilization with digitonin was used to examine the release of cytochrome c from mitochondria into cytosol (23). Briefly, cells treated with different concentrations of SL11144 for the desired exposure time were harvested by trypsinization, washed with phosphate-buffered saline (PBS) and subsequently incubated in 100µl of permeabilization buffer (210 mM D-mannitol, 70 mM sucrose, 10 mM HEPES, 5 mM succinate, 0.2 mM EGTA, 100 µg/ml digitonin, pH 7.2) for 5 min. After centrifugation for 10 min at 14,000 x g, the supernatant with protein content was saved and protein concentrations were determined using the Pierce Micro Protein Assay Kit. Equal amounts of protein were fractionated on a 12 % SDS-polyacrylamide gel electrophoresis (PAGE) and analyzed by Western blot as described below.

Western Blotting. Cells treated with different concentrations of SL11144 for the desired exposure times were harvested by trypsinization and washed with phosphate-buffered saline (PBS). Cellular protein was isolated using the protein extraction buffer containing 150 mM NaCl, 10 mM Tris, pH 7.2, 5 mM EDTA, 0.1% Triton X-100, 5% glycerol, 2% SDS. Protein concentrations were determined using Pierce Micro Protein Assay Kit. Equal amounts of

proteins (50 μg/lane) were fractionated on a 12 % SDS-polyacrylamide gel electrophoresis (PAGE) and transferred to PVDF membranes. The membranes were incubated with primary antibodies against caspase 3, PARP, Bcl-2, Bax, caspase 8, caspase 9, cytochrome c, c-jun, c-fos, or Fas Ligand (1:2000; Santa Cruz Biotechnology, Santa Cruz, CA). After washing with PBS, the membranes were incubated with peroxidase-conjugated goat anti-mouse or anti-rabbit secondary antibody (1:3000; DAKO Corporation, Carpinteria, CA) followed by enhanced chemiluminescent staining using the enhanced chemiluminescence system (Amersham Biosciences). Actin was used to normalize for protein loading.

RESULTS

Inhibition of growth by SL11144. The sensitivity of three human breast cancer cell lines (MDA-MB-231, MDA-MB-435, and MCF7 cells) to the newly synthesized polyamine analogue SL11144 (Fig. 1) was assessed by using a MTT cellular survival assay. These cells were chosen as they represent hormone-responsive (MCF7) and –unresponsive (MDA-MB-231 and MDA-MB-435) human breast cancer cell lines. All three cell lines exhibited time- and concentration-dependent growth inhibition by SL11144 (Fig. 2). The IC_{50} values for MDA-MB-231 and MCF7 cells are about 5 μM for a 72 h treatment and 0.5-0.75 μM for a 96 h treatment. MDA-MB-435 cells were more sensitive to SL11144 with IC_{50} values of 2.5 μM for 24 h and 0.25-0.5 μM for 48 h drug exposures.

Regulation of intracellular polyamine pools and metabolic enzymes by SL11144. To address whether the observed growth inhibitory effects of SL11144 in human breast cancer cells reflect its effects on the polyamine metabolic pathway, intracellular SL11144 accumulation, polyamine pools (putrescine, spermidine, spermine) and regulatory enzyme (SSAT, ODC) activities were assessed. As shown in Table 1, the accumulation rates of SL11144 in the three cell lines are similar after exposure of cells to 10 μM SL11144 for 24 h. SL11144 decreased putrescine, spermidine and spermine levels in both MDA-MB-231 cells and MCF7 cells. Spermidine was decreased and spermine was slightly increased by SL11144 in MDA-MB-435 cells. ODC activity was significantly inhibited and SSAT activity was modestly increased in all three cell lines.

SL11144 induces apoptotic cell death. To determine if observed SL11144-induced decrease in growth rate was a result of apoptosis, DNA fragmentation assays were performed. DNA ladders isolated from untreated and SL11144-treated cells were processed by agarose gel electrophoresis to detect the typical oligonucleosomal DNA fragmentation. The results (Fig. 3) indicate that SL11144 induces DNA fragmentation in all three human breast cancer cell lines, but the time and dose required for the induction of apoptosis varied by cell type. DNA fragmentation was clearly detected only after a 96 h exposure to SL11144 in MDA-MB-231 and MCF7 cells, but was detectable in 12 h in MDA-MB-435 cells. A minimum concentration of 5 μM is required for SL11144 to induce DNA fragmentation in MDA-MB-231 and MDA-MB-435 cells, but MCF7 cells are more sensitive in that DNA fragmentation was observed with 0.1-0.25 μM for 96 h.

The effect of SL11144 on cell morphology was also investigated. Both control and SL11144 treated cells were stained with the fluorescent dye Hoechst 33258 and visualized by fluorescence microscopy. Typical morphological changes of apoptosis including chromatin condensation and nuclear fragmentation were observed in all three treated cell lines (Fig. 4B), but not in the untreated control cells (Fig. 4A). Taken together, the DNA fragmentation and fluorescence results suggest that SL11144 induces apoptotic cell death in all three human breast cancer cell lines.

Effect of SL11144 against human breast cancer MDA-MB-231 xenografts. The *in vivo* effect of SL11144 was evaluated using human breast cancer MDA-MB-231 xenografts in athymic nude mice. By twenty days following tumor cell inoculation, most mice had developed palpable tumors. The average tumor size was approximately 0.2 cm^3. Mice were

randomized into treatment (n=12) and control (n=9) groups. SL11144 (10 mg/kg/mouse/day) was administered via intraperitoneal injections for 5 consecutive days (day 20-24, day 38-42, day 59-63) for a total of 3 cycles. The effects of SL11144 on the growth of the MDA-MB-231 xenografts are depicted in Fig. 5A. Treatment with SL11144 clearly inhibited the tumor growth within the first cycle of treatment and throughout the whole course of treatment. On day 36, all the mice in the control group were sacrificed because of excess tumor burden. Two SL11144-treated mice were sacrificed on day 56 and another two were sacrificed on day 67 due to excess tumor burden. During the course of treatment there was no significant decrease in mouse body weights (Fig. 5B) and no mouse died from toxicity during treatment. In addition, no apparent histological abnormality in livers and kidneys from treated animals was observed (data not shown). These results indicated that the treatment of SL11144 had significant *in vivo* growth-suppression efficacy against MDA-MB-231 cells with no overt toxic effects.

Effects of SL11144 on apoptosis related proteins. Several apoptosis-associated genes or proteins have been shown to play critical roles in regulating apoptosis. These include caspases, bcl-2 family members, Fas ligand, cytochrome c, and PARP (24-28). To determine if these proteins are involved in the mediation of SL11144-induced cell death in human breast cancer cells, we examined their expression by Western blotting. In MDA-MB-231 cells, as shown in Fig. 6A, treatment with 10 μM SL11144 decreased the amount of caspase-3 protein by 96 h treatment, but no cleaved, active caspase-3 or its downstream target, PARP was detected. We next examined whether two upstream proteases, caspase-9 and caspase-8, were affected by SL11144 in MDA-MB-231 cells. SL11144 treatment increased the cleavage

of caspase-9 after 72 h, while caspase-8 was essentially undetectable under the conditions examined. Cytochrome c release from mitochondria was enhanced at 48 h and returned to its baseline level thereafter. Further, Bcl-2 protein was down-regulated and Bax was up-regulated by SL11144 beginning at 24 h. Finally, expression of Fas Ligand was increased by SL11144 after 48 h. The changes observed precede DNA fragmentation, which was not observed until 96 h of treatment.

Effects of SL1144 on apoptotic protein expressions were further assessed in MCF7 cells. Our results (Fig. 6B) confirmed previous studies (29) that caspase 3 is not expressed in MCF7 cells. The activities of caspase-8, caspase-9 and PARP were not affected by SL11144. SL11144 did not change Bcl-2 protein levels, while Bax expression was minimal. No release of cytochrome c was observed in SL11144 treated MCF7 cells. However, Fas Ligand level was increased by SL11144 after 48 h of treatment. These data suggest that SL11144 may induce apoptosis in MCF7 cells through caspase- and cytochrome c release-independent pathways. Another possibility is that the DNA fragmentation and apoptotic morphologic changes noted in MCF7 cells are induced directly by SL11144. Many polyamine analogues bind strongly to DNA, and are capable of inducing structural changes in chromatin (6).

In contrast to the above results in MCF7 and MDA-MB-231 cells, SL11144 treatment of MDA-MB-435 cells led to caspase-3 activation and cleavage of PARP within 12 h of drug exposure (Fig. 6C). Also caspase-8 was activated and the proform of caspase-9 was completely cleaved by 48 h exposure. Although Bcl-2 expression did not change and Bax expression was essentially undetectable, cytochrome c was released into cytoplasm from mitochondria by 12 h. Fas Ligand expression was induced at 48 h. These results suggest that both caspase and mitochondrial pathways are activated by SL11144 in MDA-MB-435 cells.

The time course of caspase activation and cytochrome c release parallels the course of DNA fragmentation, which was detected at 12 h of SL11144 treatment.

SL11144 up-regulates c-Jun and c-Fos in MDA-MB-231 and MDA-MB-435 cells. Since the effects of SL11144 on apoptotic pathways varied greatly between different human breast cancer cell lines, we examined its impact on other important apoptosis related factors, particularly c-Jun and c-Fos. Both c-Jun and c-Fos are important members of the AP-1 (Activator Protein-1) transcription factor family, which plays a critical role in regulating transcription of a variety of genes involved in growth, differentiation, apoptosis, etc. In MDA-MB-231 cells, SL11144 phosphorylated c-Jun after 48 h but did not alter the protein expression of either c-Jun or c-Fos (Fig. 7A). In MCF7 cells, no obvious changes in c-Jun and c-Fos were observed (Fig. 7B). In contrast, SL11144 significantly induced the phosphorylation of c-Jun and enhanced the protein level of c-Jun and c-Fos in MDA-MB-435 cells within 6-12 h (Fig. 7C). To study whether SL11144-enhanced c-Jun and c-Fos protein expressions led to the increased nuclear localization of these protein in MDA-MB-435 cells, the subcellular localization of c-Jun and c-Fos were examined. c-Jun was induced and expressed largely in the nucleus, whereas c-Fos was induced in both the cytoplasm and the nucleus (Fig. 7D). These results imply that up-regulation of the AP-1 family may play an active role in the mediation of SL11144-induced growth inhibition in MDA-MB-231 and in MDA-MB-435 cells.

DISCUSSION

Previous studies in our laboratory demonstrated that first and second generations of N-acetyl substituted polyamine analogues could inhibit growth and induce apoptosis in MCF7 and in other human breast cancer cell lines (3, 9, 30). However a phase II clinical trial of one early polyamine analogue, N^1, N^{11}-diethylnorspermine (DENSPM), was not effective as a single agent in women with advanced breast cancer (31). Recently, a group of new polyamine analogues designated as oligoamines has been developed (17). Oligoamines were synthesized with longer chains than natural cellular polyamine molecules that occur in mammalian cells, and are effective against a variety of proliferating cells (17). In this study we demonstrate that one of the lead oligoamine compounds, SL11144, significantly inhibits the growth of, and induces programmed cell death in, human breast cancer cells. SL11144 induced DNA fragmentation and typical apoptotic morphological changes in both hormone-responsive (MCF7) and hormone-unresponsive (MDA-MB-231 and MDA-MB-435) breast cancer cell lines. It appears that there is no apparent relationship between hormone receptor status and cytotoxic effects of SL11144. SL11144 also inhibits the growth of MDA-MB-231 xenografts in nude mice without apparent toxicity as manifested by stable body weight.

SL11144-induced apoptosis, based on morphologic and DNA fragmentation criteria, was not detected until 96 h treatment in MDA-MB-231 and in MCF7 cells, but 12 h treatment with SL11144 resulted in apoptosis in MDA-MB-435 cells. Although the mechanisms of differential susceptibility among tumor cells to polyamine analogue-induced cell death are unclear, this could reflect varied effects on apoptotic pathway members including caspases, bcl-2 family members, cytochrome c and Fas-ligand, which have been demonstrated to play critical roles in regulating programmed cell death (24-27). Caspases have been characterized

as the effectors and executioners of apoptosis, and caspase-3 is a critical downstream apoptotic effector that cleaves specific substrates like PARP. The observation that caspase-3 activation was followed by PARP cleavage in MDA-MB-435 cells indicates that caspase-3 may play a key role as an important executioner in SL11144-induced apoptosis in this cell line. However, the failure of SL11144 to activate caspase-3 in MDA-MB-231 cells and the absence of caspase-3 expression in MCF7 suggest that other factors or pathways can also function as apoptotic effectors in these two cell lines.

Mitochondria can be induced to release cytochrome c in response to many anticancer drugs and to other stresses by the opening of channels on the outer mitochondrial membrane (34). Release of cytochrome c activates the caspase adaptor, caspase-9, which then activates downstream caspases like caspase-3 and caspase-8 (35). Our studies found that cytochrome c release was transiently enhanced by SL11144 with a 48 h drug exposure in MDA-MB-231 cells, while it was rapidly and consistently induced in MDA-MB-435 cells. In both cell lines, time-dependent activation of caspase-9 was observed, but caspase-8 activation was only seen in MDA-MB-435 cells. However, in MCF7 cells, SL11144 has no effect on cytochrome c release or on caspase-8 or –9 activation. The simultaneous activation of both caspase cascades and of the mitochondrial pathway in MDA-MB-435 cells by SL11144 might explain why cell death was more rapidly induced in these cells than in MDA-MB-231 or MCF7 cells.

Members of the Bcl-2 family play a central role in regulating the mitochondrial pathway of apoptosis. More than 20 Bcl-2 family members have been identified to date, including anti-apoptosis members (Bcl-2, Bcl-X_L, Bcl-W, Bcl-G, Mcl-1, etc) and pro-apoptosis members (Bax, Bak, Bok, Bad, Bid, Bik, Bim, Bcl-Xs, etc) (32-36). In response to various

stimuli and stresses, Bcl-2 family proteins usually translocate to the outer mitochondrial membrane and modulate membrane permeabilization, leading to the release of cytochrome c. SL11144 decreased Bcl-2 and increased Bax expression in MDA-MB-231 cells, but did not affect Bcl-2 and Bax in MDA-MB-435 and MCF7 cells suggesting that the regulation of Bcl-2 family members by polyamine analogue is cell-type specific. Our data also demonstrate that SL11144 enhances Fas-ligand expression in all three cell lines, the only protein to be uniformly affected. The Fas/Fas-ligand (CD95-CD95 ligand) system is another critical pathway that leads to the activation of apoptotic machinery. Binding of Fas-ligand to Fas and to other death receptors results in receptor trimerization, recruitment of adaptor protein to the cytoplasmic death domain, and activation of a series of downstream apoptotic events (38, 39). Recent studies have shown that over-expression of Fas-ligand can lead to suicidal or fratricidal destruction in melanoma and leukemia cells via autocrine or fratricidal interactions between Fas-ligand and Fas (40, 41). Up-regulation of Fas-ligand level by SL11144 in all three human breast cancer cell lines implies that activation of Fas/Fas-L system might be a common mechanism for the cell death induced by SL11144.

We further investigated if other important upstream regulatory or signaling events were involved in the mediation of SL11144-induced growth inhibition and apoptosis. SL11144 induces expression and phosphorylation of c-Jun, an important member of the AP-1 family, in both MDA-MB-231 and MDA-MB-435 cells. It also significantly increased the protein expression of another important AP-1 family member, c-Fos, after 12 h in MDA-MB-435 cells. Nuclear extraction analysis showed that c-Jun protein was located largely in the nucleus, where it can potentially play an active role in mediation of a wide range of gene expressions. However, neither c-Jun nor c-Fos levels or phosphorylation status were affected

by SL11144 treatment in MCF7 cells. Jun N-terminal kinase (JNK) signaling and AP-1 transcription factors have been implicated in the regulation of cell proliferation, differentiation and apoptosis (42). The proapoptotic targets of c-jun include Fas-ligand, TNF-α, c-Myc, p53, and members of the bcl-2 family (43-47). The activation of c-jun in MDA-MB-231 and MDA-MB-435, but not in MCF7 cells suggests that JNK/AP-1 and the upstream regulator MAP kinase family might be a major polyamine analogue response pathway in some but not all breast cancer cell lines.

The intracellular polyamines are highly regulated by several polyamine metabolic enzymes. Ornithine decarboxylase (ODC), the first and rate-limiting step of polyamine biosynthesis, increases levels of polyamines in cells during rapid proliferation or differentiation (6). High expression of ODC characterizes some cancers including breast cancer. As a result, there has been extensive effort to design compounds, which can inhibit ODC activity in tumor cells. DFMO, an irreversible inhibitor of ODC, has proven to be effective in inhibiting growth in several *in vitro* and *in vivo* tumor models (6, 49). In this study, SL11144 significantly inhibits ODC activities in all three breast cancer cell lines tested. However, the activity of another critical polyamine metabolic enzyme, SSAT, was only modestly up-regulated by SL11144 exposure, suggesting that its activity is not responsible for the observed cytotoxic response. The effect of SL11144 on natural polyamine levels was variable. SL11144 treatment led to a decrease in all natural polyamines in MDA-MB-231 and MCF7 cells and had inconsistent effects in MDA-MB-435 cells. This suggests that SL11144 effects are not solely a function of its effect on polyamine pools.

In summary, a newly developed polyamine analogue, SL11144, exhibits significant inhibitory actions against human breast cancer cell growth *in vitro* and *in vivo*. Apoptotic cell

death was induced by SL11144 in a time- and dose-dependent manner. SL11144 modulated expression of apoptotic proteins in a cell-type specific manner, suggesting that multiple apoptotic pathways might be involved in SL1144-induced apoptosis in different human breast cancer cell lines.

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LEGENDS

Figure 1. Structures of spermine and polyamine analogue SL11144.

Figure 2. SL11144 inhibits growth of human breast cancer cells in a time and dose dependent manner. MDA-MB-231 cells (A) and MCF7 cells (B) were treated with increasing concentrations of Sl11144 for 72 h or 96 h. MDA-MB-435 cells (C) were treated with increasing concentrations of Sl11144 for 24 h or 48 h. MTT assays were performed as described in *Materials and methods*. Shown are means $\pm$ SD of independent experiments performed in quadruplicate.

Figure 3. SL11144 induces internucleosomal DNA fragmentation. MDA-MB-231 cells (A) and MCF7 cells (B) were treated with increasing concentrations of SL11144 (0.1-10 μM) for 96 h or treated with 10 μM for 48, 72 and 96 hours. MDA-MB-435 cells (C) were treated with increasing concentrations of SL11144 (0.1-10 μM) for 24 hours or treated with 10 μM for 3, 6, 12, 24, and 48 hours. Cells were harvested, and fragmented DNA was extracted as described in *Materials and Methods*. Fragmented DNA was analyzed by electrophoresis in a 1.2% agarose gel containing 0.1% ethidium bromide. Each experiment was done twice with similar results.

Figure 4. Fluorescent micrographs of SL11144 treated cells. MDA-MB-231 cells, MDA-MCF7 cells and MB-435 cells were exposed to 10 μM SL11144 for the indicated times. Then cells were fixed in formaldehyde and stained with Hoechst dye 33258.

Figure 5. Effects of SL11144 in nude mice bearing MDA-MB-231 xenografts. (A) MDA-MB-231 tumors were transplanted into the flank region of nude mice. Twenty days after implantation, when tumor reached a volume of 0.1-0.2 cm^3, mice was randomly assigned to receive vehicle control or SL11144 (10 mg/kg/mouse/day) via intraperitoneal injection for 5 consecutive days in a 3-week cycle for a total of 3 cycles. Tumor volumes of mice were measured twice weekly. All the mice in control group were sacrificed on day 36 because of the excess of tumor burden. Two SL11144-treated mice were sacrificed on day 56 and two other were sacrificed on day 67 due to the excess of tumor burden. The vertical bars indicate mean tumor size in cm^3 ± SE. (B) Weights of mice were measured twice weekly. The vertical bars indicate mean mouse weight in g ± SE.

Figure 6. Effects of SL11144 on apoptosis proteins. MDA-MB-231 cells (A) and MCF7 cells (B) were treated with 10 μM SL11144 for 24, 48, 72, and 96 hours. MDA-MB-435 cells (C) were treated 10 μM SL11144 for 12, 24 and 48 hour. Equal amounts (50 μg/lane) of cellular protein were fractionated on 12 % SDS-PAGE gels and transferred to PVDF membranes followed by immunoblotting with anti-caspase 3, PARP, caspase-9, caspase-8, cytochrome c, Bcl-2, Bax, Fas-L monoclonal or polyclonal antibodies and analyzed as described in *Materials and Methods*. Actin protein was blotted as a control. Each experiment was repeated twice with similar results.

Figure 7. Effects of SL11144 on c-Jun and c-Fos. Tumor cells were treated with 10 μM SL11144 for the times indicated. Equal amounts (50 μg/lane) of whole cell (A, B, C), cytoplasmic or nuclear protein (D) were fractionated on 12% SDS-PAGE gels and

transferred to PVDF membranes followed by immunoblotting with anti-c-Jun and c-Fos polyclonal antibodies and analyzed as described in *Materials and Methods*. Actin protein was blotted as a control.

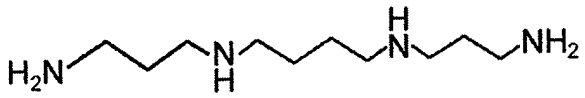
Table 1:

Table 1. *Effects of SL11144 on polyamine pools, and SSAT and ODC activities in human breast cancer cells*
Intracellular polyamine levels, SSAT activity, and ODC activity were determined as described in "Materials and Methods" following incubation of tumor cells for 24 h in the presence or absence of 10 μM SL11144.

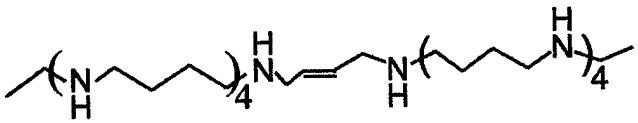
Cell lines	Treatment	SL11144 (nmol/mg protein)	Polyamines (nmol/mg protein)			SSAT activity (pmol/mg Protein/min)	ODC activity ($pMCO_2$/hr/mg)
			Put[a]	Spd	Spm		
MDA-MB-231	Control	ND	3.65	52.04	21.17	2.22	2290.98
	SL11144	2.32	ND	15.27	8.14	6.02	ND
MCF7	Control	ND	6.22	49.38	28.52	1.39	2359.10
	SL11144	4.81	0.95	58.53	16.58	14.72	6.53
MDA-MB-435	Control	ND	ND	22.03	3.24	2.73	558.79
	SL11144	3.62	ND	8.84	4.35	23.39	4.58

[a]Put, putrescine; Spd, spermidine; Spm, spermine; ND, not detected.
Values represent the means of duplicate determinations.

Figure 1:



Spermine



SL11144

Figure 2:

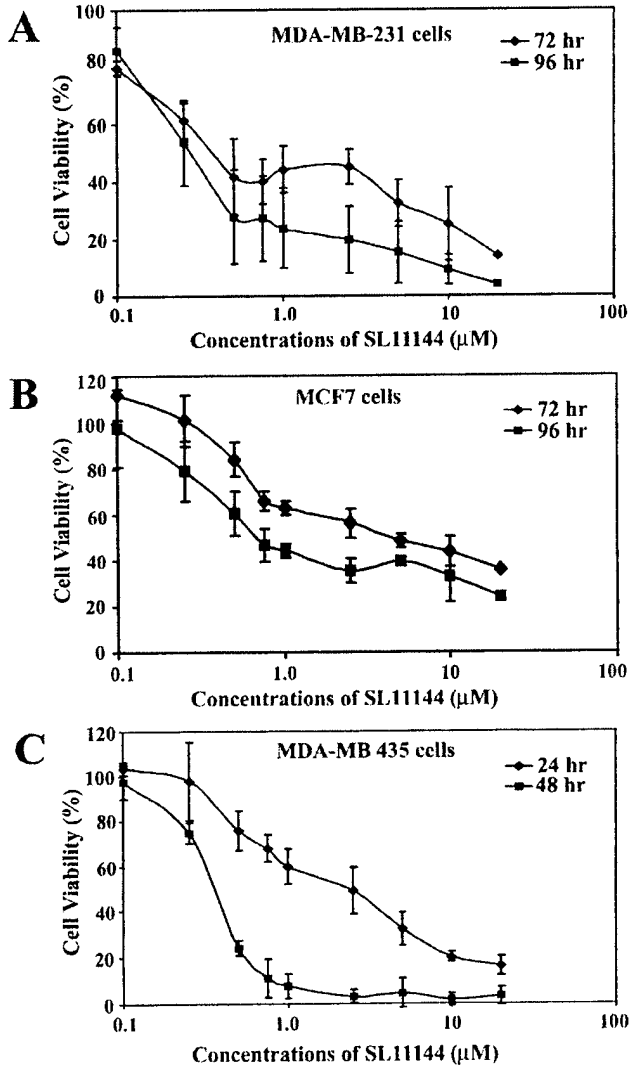

A
MDA-MB-231 cells
72 hr
96 hr
Cell Viability (%)
Concentrations of SL11144 (μM)
B
MCF7 cells
72 hr
96 hr
Cell Viability (%)
Concentrations of SL11144 (μM)
C
MDA-MB 435 cells
24 hr
48 hr
Cell Viability (%)
Concentrations of SL11144 (μM)

Figure 3:

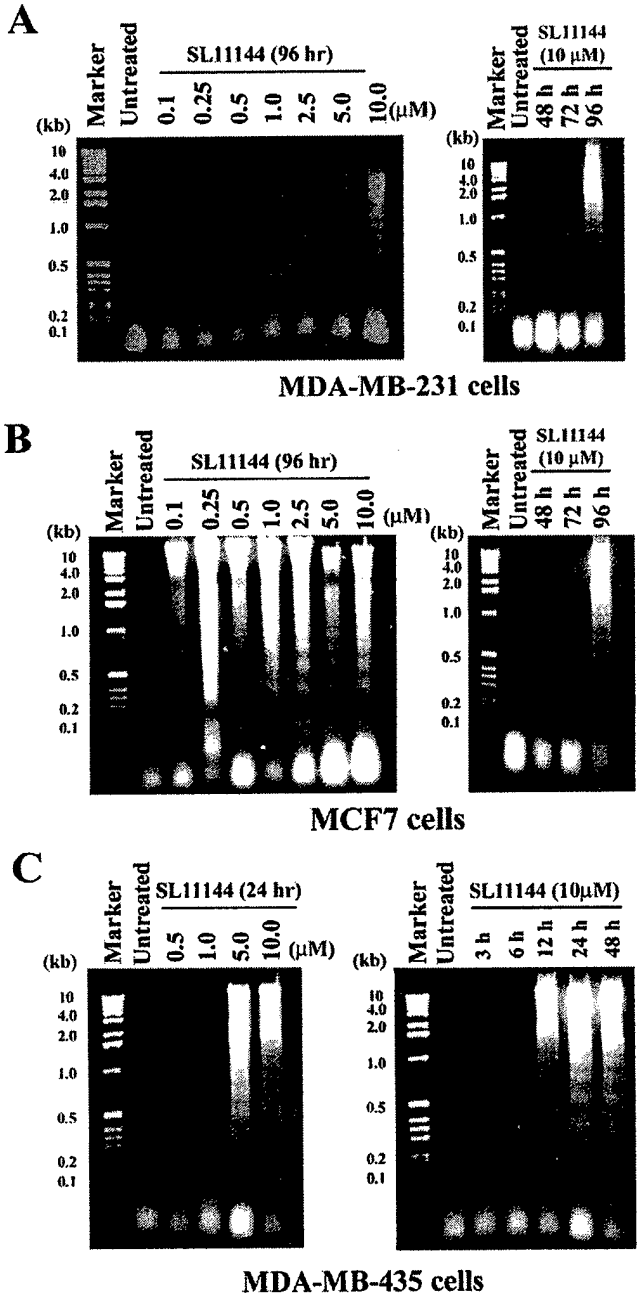

A
Marker
Untreated
SL11144 (96 hr)
0.1
0.25
0.5
1.0
2.5
5.0
10.0
(μM)
(kb)
10
4.0
2.0
1.0
0.5
0.2
0.1
Marker
Untreated
SL11144 (10 μM)
48 h
72 h
96 h
MDA-MB-231 cells
B
MCF7 cells
C
SL11144 (24 hr)
SL11144 (10μM)
3 h
6 h
12 h
24 h
48 h
MDA-MB-435 cells

Figure 4:

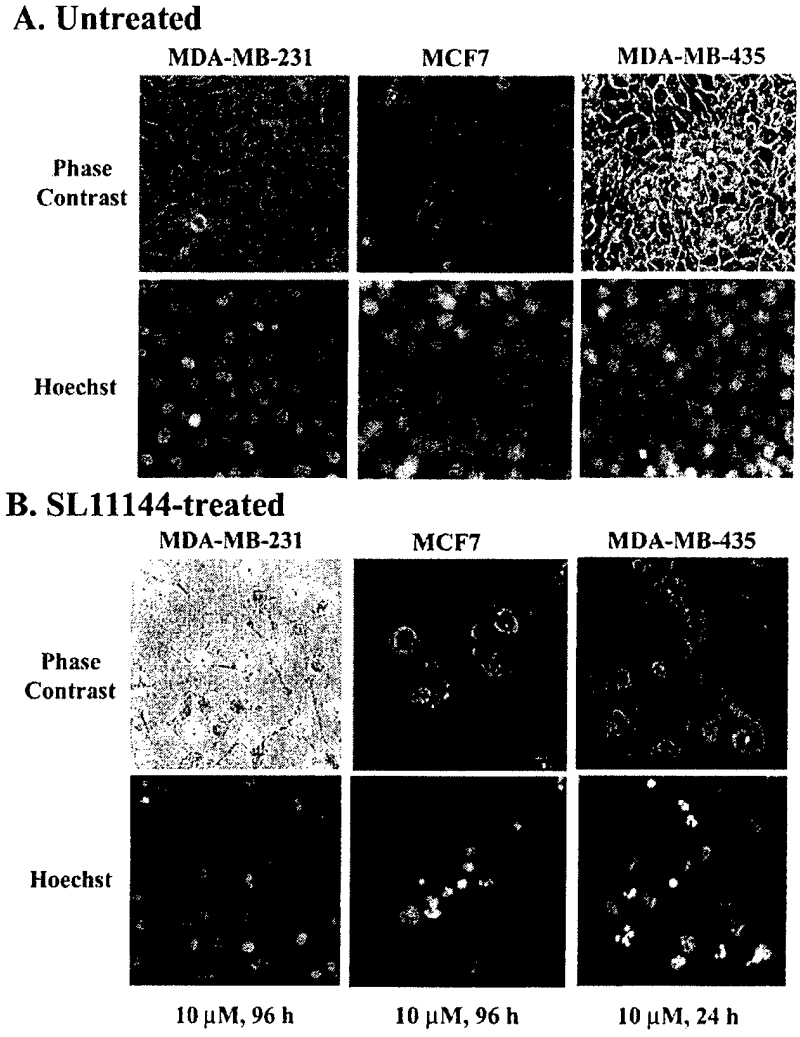

A. Untreated
MDA-MB-231
MCF7
MDA-MB-435
Phase Contrast
Hoechst
B. SL11144-treated
MDA-MB-231
MCF7
MDA-MB-435
Phase Contrast
Hoechst
10 μM, 96 h
10 μM, 96 h
10 μM, 24 h

Figure 5:

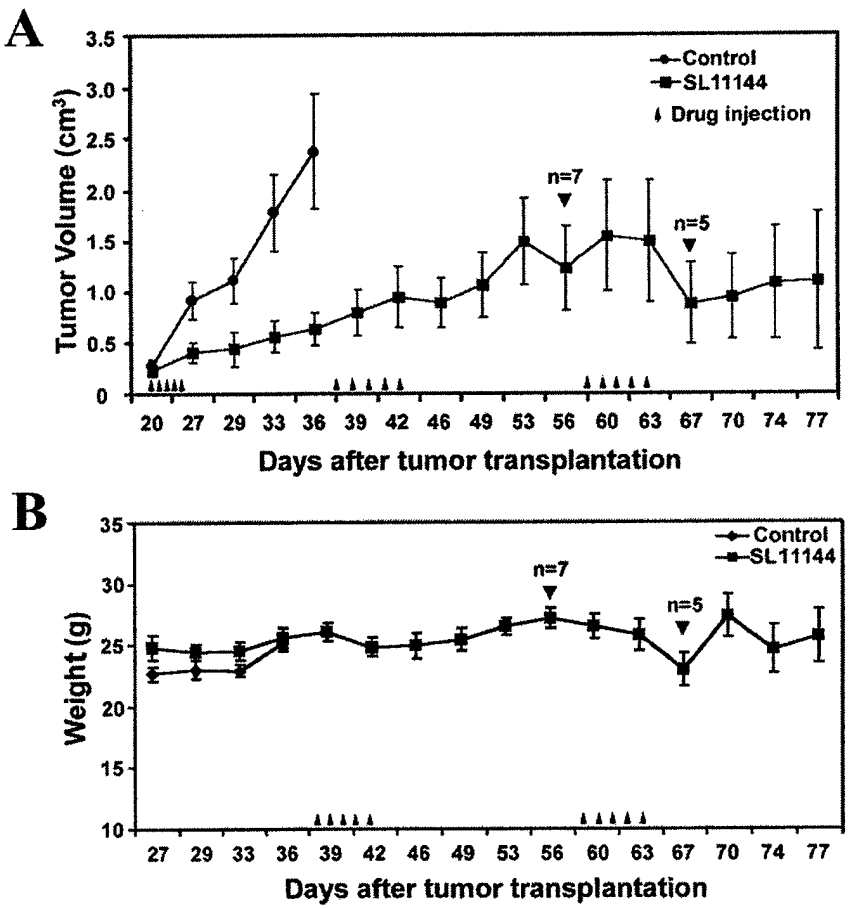

A
Tumor Volume (cm³)
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0
Control
SL11144
Drug injection
n=7
n=5
20 27 29 33 36 39 42 46 49 53 56 60 63 67 70 74 77
Days after tumor transplantation
B
Weight (g)
35
30
25
20
15
10
Control
SL11144
n=7
n=5
27 29 33 36 39 42 46 49 53 56 60 63 67 70 74 77
Days after tumor transplantation

Figure 6:

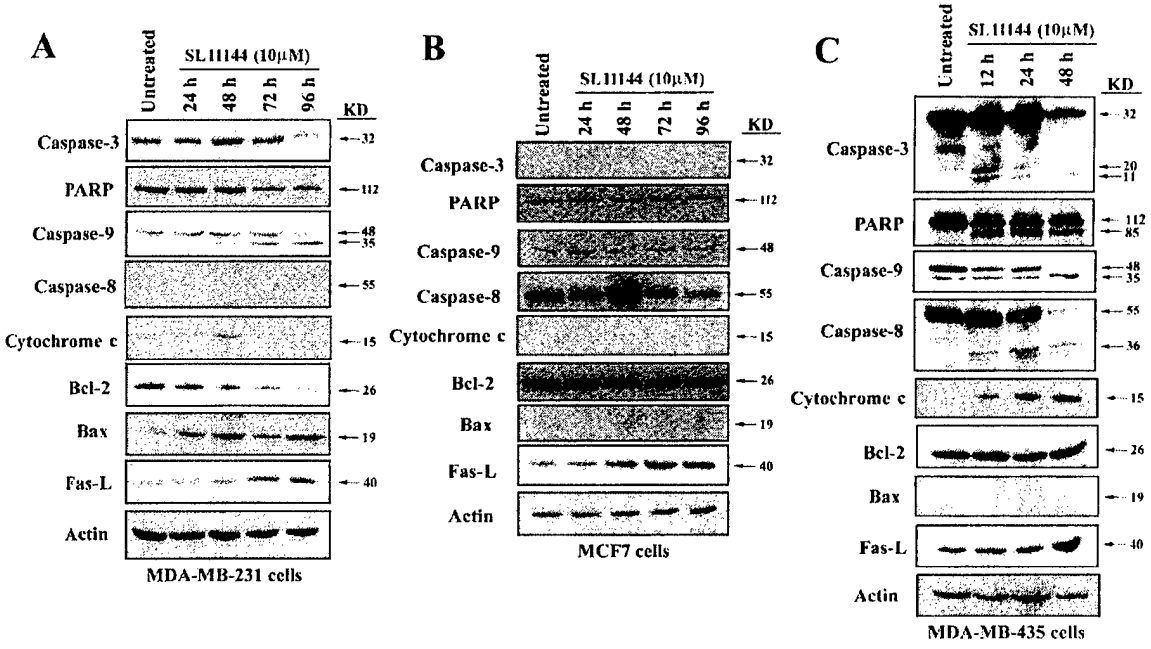
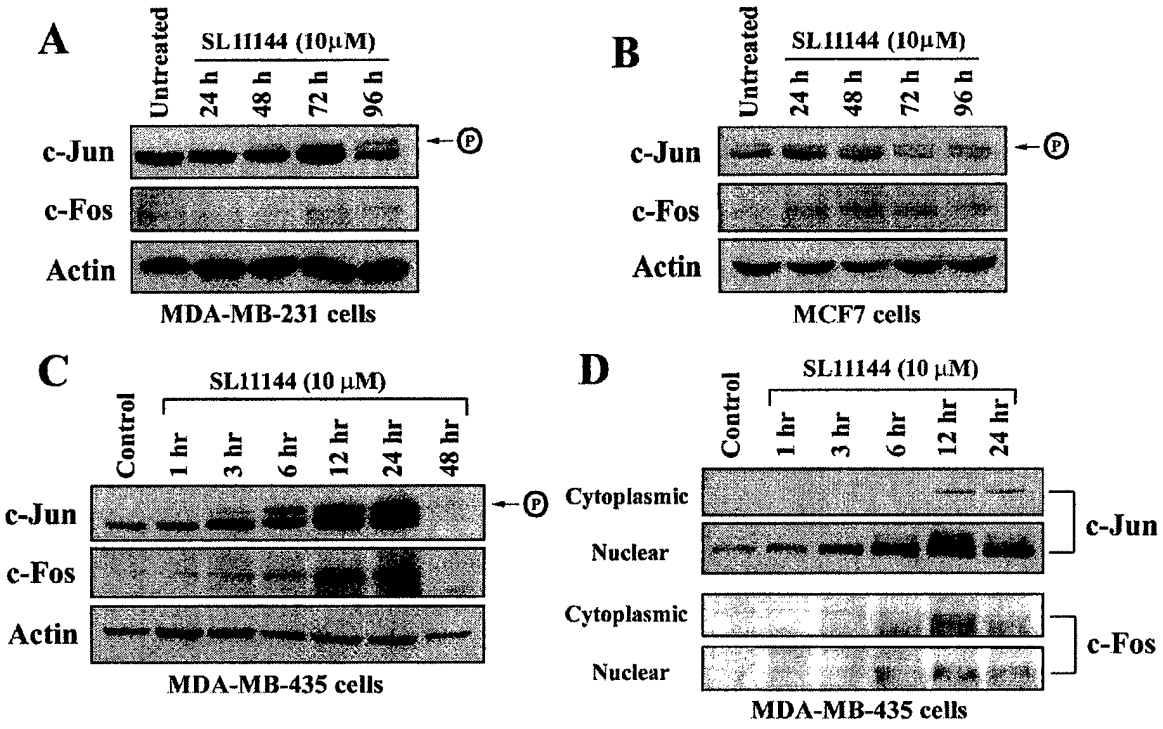

A
Untreated
SL11144 (10μM)
24 h
48 h
72 h
96 h
KD
Caspase-3
PARP
Caspase-9
Caspase-8
Cytochrome c
Bcl-2
Bax
Fas-L
Actin
32
112
48
35
55
15
26
19
40
MDA-MB-231 cells
B
Untreated
SL11144 (10μM)
24 h
48 h
72 h
96 h
KD
Caspase-3
PARP
Caspase-9
Caspase-8
Cytochrome c
Bcl-2
Bax
Fas-L
Actin
32
112
48
55
15
26
19
40
MCF7 cells
C
Untreated
SL11144 (10μM)
12 h
24 h
48 h
KD
Caspase-3
PARP
Caspase-9
Caspase-8
Cytochrome c
Bcl-2
Bax
Fas-L
Actin
32
20
11
112
85
48
35
55
36
15
26
19
40
MDA-MB-435 cells

Figure 7:


A
Untreated
SL11144 (10μM)
24 h
48 h
72 h
96 h
c-Jun
c-Fos
Actin
P
MDA-MB-231 cells
B
Untreated
SL11144 (10μM)
24 h
48 h
72 h
96 h
c-Jun
c-Fos
Actin
P
MCF7 cells
C
Control
SL11144 (10 μM)
1 hr
3 hr
6 hr
12 hr
24 hr
48 hr
c-Jun
c-Fos
Actin
P
MDA-MB-435 cells
D
Control
SL11144 (10 μM)
1 hr
3 hr
6 hr
12 hr
24 hr
Cytoplasmic
Nuclear
c-Jun
Cytoplasmic
Nuclear
c-Fos
MDA-MB-435 cells

437 A phase I study of diethylnorspermine (DENSPM) in previously treated patients with metastatic breast cancer (MBC).

Wolff AC, Bowling MK, DeClue C, Armstrong DK, Fetting JH, Casero, Jr. RA, Davidson NE. The Johns Hopkins Oncology Center, Baltimore, MD.

Polyamines are ubiquitous intracellular polycationic molecules essential for cell growth and differentiation. Eflornithine (DFMO) irreversibly inhibits ornithine decarboxylase (ODC), the first enzyme in the polyamine biosynthetic pathway, but causes reversible ototoxicity. Several polyamine analogues downregulate ODC while inducing the catabolic enzyme spermidine/spermine N1-acetyltransferase (SSAT). These analogues do not substitute for the natural polyamines, and ultimately cause depletion of intracellular pools and cell growth inhibition. The analogue N1,N11-diethylnorspermine (DENSPM) significantly inhibits the growth of multiple breast cancer cell lines, irrespective of hormone receptor status. A phase I study at Johns Hopkins identified gastrointestinal symptoms (abdominal pain with CT scan changes suggestive of small bowel inflammation) as the dose-limiting toxicity using a 15'-minute infusion days 1-5 repeated every 21 days (Ettinger et al., Proc ASCO 1998). Toxicities seen with other administration schedules (e.g., twice-daily infusions) include paresthesias, neuromotor deficit, and transient creatinine elevation. We present interim results of a follow-up study, an open-label single-center phase II study of DENSPM in pts. with MBC. Pts. were treated with the maximum tolerated dose of 100 mg/m2 IV days 1-5 repeated every 21 days based on our previous phase I study. The primary study objective is to estimate if at least 20% of pts. are progression free at 4 months. The overall accrual goal is 34 pts. (30 evaluable) with a 2-stage design. The second stage of accrual will proceed if 2 or more among the first 15 evaluable pts. are progression-free at 4 months. To date, 16 pts (median age 52, range 34-65; PS 1, range 0-1) received 39 cycles of DENSPM (median 2, range 1-4). As expected, no hematologic toxicity was detected. All pts. developed transient perioral paresthesias during drug infusion. There were five episodes of gr. 1 abdominal pain toxicity. All pts received 5-HT3 antagonists; none had nausea > gr. 1. One pt with Cy 2 gr. 3 toxicity (CT changes showing jejunal wall thickening) was taken off study. There were no other gr. 3 treatment-related toxicities. Thus far, best response observed is stable disease. Preliminary results indicate that this dose/schedule is quite tolerable. Initial assessment of efficacy will occur upon completion of the first stage of accrual.

#449 Conformationally constrained polyamine analogues and oligoamines inhibit growth and induce apoptosis in human breast cancer cells. Yi Huang, Erin R. Hager, Dawn L. Phillips, Amy Hacke, Benjamin Frydman, Aldonia L. Valasinas, Venodhar K. Reddy, Laurence J. Marton, Robert A. Casero, and Nancy E. Davidson. *Dept. of Oncology, Johns Hopkins University, School of Medicine, Baltimore, MD, and SLIL Biomedical Corp., Madison, WI.*

Polyamine analogues have demonstrated considerable promise against many important solid tumor models including breast cancer. However, the precise mechanism of anti-tumor activities of polyamine analogues is not well understood. We have evaluated and characterized seven representatives of a new generation of conformationally constrained polyamine analogues and oligoamines that exhibited significant growth inhibitory effects against human breast cancer cell lines including MCF7 and MDA-MB-231 cells. The polyamine analogue concentrations required for 50% growth inhibition (IC50) were generally less than 1 microM for 96 h. Modest induction of polyamine catabolic enzyme spermidine/spermine N1-actetyltransferase (SSAT) and polyamine pool depletion was observed by 24h. For a majority of compounds tested, internucleosomal DNA fragmentation was determined to be time and concentration dependent. Further analyses indicate that the intrinsic mitochondrial apoptotic pathway is activated by polyamine analogues in MDA-MB-231 cells, but not in MCF7 cells, suggesting that different mechanisms and pathways are involved in polyamine analogue-induced apoptosis. In addition, polyamine analogues significantly inhibited the growth of transplanted MDA-MB-231 cells in nude mice. These results suggest that the newly synthesized polyamine analogues show effective antitumor action against human breast cancer cells. [Supported by NIH Grants P50CA88843, CA51085, Army DOD Grant DAMD 17-99-1-9242, and DAMD17-00-1-0301]